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The effects of heat stress on energetic metabolism and insulin homeostasis

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The effects of heat stress on energetic metabolism and insulin homeostasis

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DEDICATION

A mi familia. En especial a mis padres, porque sin ellos no sería. Por compartir mis alegrías y apoyarme en cada fracaso, especialmente durante los últimos cuatro años en los que habéis hecho que la distancia no existiera. Os quiero mucho.

Toyita

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

5'D	Hepatic 5'-Deiodinase type I
ACC	Acetyl CoA Carboxylase
AKT	Protein Kinase B
AMPK	Adenosine Monophosphate Activated Protein Kinase
APP	Apparent Permeability Coefficient
AT	Adipose Tissue
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BCA	Bicinchonic Acid Assay
BHB	β-Hydroxybutirate
BUN	Blood Urea Nitrogen
BW	Body Weight
cAMP	Cyclic Adenosine Monophosphate
CWP	Colostral Whey Protein
CPT-1	Carnitine Palmitoyl Transferase I
D	Day
EC	Epinephrine Challenge
FI	Feed Intake
FITC	Fluorescein Isothiocyanate
GLP-1	Glucagon Like Peptide I
GSIS	Glucose Stimulated Insulin Secretion
GTT	Glucose Tolerance Test
H	Hour
HEC	Hyperinsulinemic Euglycemic Clamp
HIF1	Hypoxia-Inducible Factor I
HSP	Heat Shock Protein
HS	Heat Stress
HSL	Hormone Sensitive Lipase
IRS	Insulin Receptor Substrate
ITT	Insulin Tolerance Test
JNK	C-Jun Amino Terminal Kinase
LBP	Lipopolysaccharide Binding Protein
LD	Longissimus Dorsi
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
mTOR	Mammalia Target of Rapamycin
NEFA	Non-Esterified Fatty Acids
P	Period
PDE	Phosphodiesterase
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PDK	Phosphatidylinositol 3,4,5-Triphosphate-Dependent Protein Kinase
PFTN	Pair-Fed Thermoneutral
PI3K	Phosphatidylinositol 3-Kinase

PIP2	Phosphatidylinositol 4,5-Triphosphate
PIP3	Phosphatidylinositol 3,4,5-Triphosphate
PKA	Protein Kinase A
PP1	Protein Phosphatase 1
ROGI	Rate of Glucose Infusion
RR	Respiration Rate
SH2	Src Homology 2
TCA	Tricarboxylic Acid Cycle
TER	Transepithelial Electrical Resistance
TLR4	Toll-like Receptor 4
TN	Thermoneutral
TR	Rectal Temperature
WP	Milk Whey Protein
ZNAA	Zinc Amino Acid Complex

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ABSTRACT

Heat stress (HS) is a major environmental hazard for human health and animal agriculture. Changes in metabolism and specifically altered insulin action appear to be critical for the adaptation and ultimately survival to a severe heat load. However, our knowledge of the physiological and metabolic consequences of HS is woefully insufficient. Understanding the biology of HS is critical in order to develop treatment protocols and mitigation strategies against its deleterious effects on both humans and livestock. Interestingly, HS elicits a metabolic profile that somewhat resembles models of endotoxemia/sepsis. Moreover, HS negatively affects intestinal health and the subsequent increase in the permeability to luminal pro-inflammatory molecules might be the link between both models. Therefore, alleviating the effects of HS on the intestinal barrier function may have the potential to prevent or ameliorate its impact on animal health and productivity.

Studies conducted for this dissertation utilized *sus scrofa* as the model, due to the pig's relevance in both animal agriculture and the biomedical field. Study 1 (chapter 2) was conducted to determine the temporal effects of HS on basal and stimulated metabolism. Results indicated that HS decreases both basal and adrenergic-induced adipose tissue mobilization. Heat stress increased basal insulin secretion and decreased circulating thyroid hormones. Early heat exposure decreased glucose disposal in response to a glucose tolerance test, which might suggest increased whole body insulin resistance. However when considering the glucose and insulin responses together, HS pigs required less insulin compared to controls in order to dispose of a similar amount of glucose, which would actually indicate increased insulin sensitivity.

Based on the conflicting results obtained in study 1 regarding insulin action, in study 2 (chapter 3) we aimed to define the effects of HS on whole-body insulin action. For this purpose

we performed a hyperinsulinemic euglycemic clamp and concluded that, relative to controls, HS pigs required a greater rate of glucose infusion to maintain euglycemia. Further, molecular markers of insulin signaling prior and after insulin stimulation, suggested that the skeletal muscle is partially responsible for the increase in insulin-stimulated glucose uptake observed during HS.

Heat stress has deleterious effects on intestinal health that may ultimately mediate its effects on energetic metabolism and productivity. Studies 3 and 4 (chapters 4 and 5) objectives were to establish the feasibility of dietary interventions in order to mitigate HS-induced intestinal barrier dysfunction. Previous research had demonstrated that dietary zinc and whey protein improve intestinal health in a variety of bowel disease models, which led us to hypothesize that their supplementation would alleviate the negative effects of HS on intestinal permeability. Results from these studies indicated that supplemental zinc improves aspects of small intestinal integrity during severe HS. In contrast, the tested dairy products did not mitigate the deleterious consequences HS has on intestinal barrier function.

In conclusion, studies 1 and 2 demonstrated that HS markedly alters both lipid and carbohydrate metabolism. Specifically, HS decreases adipose tissue mobilization and increases whole-body insulin sensitivity. In addition, studies 3 and 4 proved that dietary interventions aimed to improve intestinal integrity during HS are plausible. A better understanding of the relative contribution of the intestinal barrier dysfunction to the heat-induced effects on metabolism and ultimately on nutrient partitioning is a prerequisite for designing targeted strategies to mitigate the consequences of HS on human health and animal productivity.

CHAPTER 1: LITERATURE REVIEW

Impact of Heat Stress

Animal agriculture

Heat stress (HS) negatively impacts animal agriculture, not only by jeopardizing animal welfare, but also by decreasing production efficiency. The economic losses derived from high ambient temperatures are estimated to be over $2 billion a year in the US alone and are the result of decreased production (decreased milk yield, egg laying, growth), altered carcass composition, depressed reproduction, increased veterinary cost, and mortality (St-Pierre *et al.* 2003; Baumgard and Rhoads 2013). For instance, a 2006 California heat wave resulted in the deaths of more than 30,000 dairy cows (California Department of Food and Agriculture 2006), and a recent heat wave in Iowa killed at least 4,000 head of beef cattle (Drovers Cattle Network 2011). Heat stress is a global problem, even in temperate areas of the world like Canada, where 40% of the summer days are considered thermally stressful for agriculturally relevant species (Ominski *et al.* 2002). However, in these temperate regions, HS is primary an issue during just the summer months, while in tropical areas it can be a year-round problem. At these latitudes, commercial breeds perform poorly due to compromised thermotolerance associated with increased productivity and metabolic heat production (Brown-Brandl *et al.* 2003). In contrast, local or indigenous breeds are well acclimated to high temperatures but their efficiency and overall productivity is typically inferior (Renaudeau *et al.* 2012). This is troubling because the human population growth is often concentrated in these areas (Roush 1994), and the inability to efficiently produce animal protein in these regions may create a food security concern (Baumgard and Rhoads 2013). Further, the negative effects of HS on animal productivity might become more of an issue in the future if, as

some have predicted, extreme weather events and specifically heat waves increase in frequency due to climate change (Luber and McGeehin 2008). Understanding the acclimation mechanisms to HS and their consequences on animal productivity are critical in order to develop mitigation strategies.

Human impact

Excessive heat claims more human lives than all other climatic events combined (Changnon *et al.* 1996). In the USA, an estimated 1,500 heat related human deaths are reported each summer (Kalkstein and Greene 1997), but this is probably underestimated as many succumb to the long-term consequences of the heat exposure (Centers for Disease Control and Prevention 2006). In Chicago, the 1995 (Semenza *et al.* 1999) and 1999 (Naughton *et al.* 2002) heat waves claimed over 600 and nearly 100 lives, respectively; and in a 2 week long heat wave in 2003, 50,000 Europeans died (Kosatsky 2005), 15,000 of those in France alone (Pirard *et al.* 2005). Heat susceptibility is not evenly distributed by age, as the very young and elderly populations are the most vulnerable to high ambient temperature exposure (Leon and Helwig 2010a). For example, in France's 2003 heat wave, excess mortality (the mortality above what would be expected under normal circumstances) was estimated at 70% for the 75-94 year age group, and at 120% for people older than 94 years of age in France (Kosatsky 2005). The combination of heat exposure with strenuous work can result in exertional heat stroke, typically affecting athletes and occupational workers (e.g. agriculture workers, fire fighters, military personnel; Wilkins and Wheeler 2004). Other conditions can also predispose people to heat stroke, including illnesses (e.g. cardiovascular disease, infections), drug use, alcoholism, etc. (Kilbourne *et al.* 1982). Despite its high death toll, the pathology of heat-related illnesses is not well understood. Diagnosis is based on hyperthermia and central nervous system dysfunction

(e.g. confusion, delirium, seizures, or coma), and the treatment protocol consists of general cooling and rehydration (Leon and Helwig 2010a). Probably the biggest limitation in treating heat victims is not being able to anticipate long term outcomes like hypothermia, fever, disseminated intravascular coagulation, systemic inflammatory response syndrome (cytokine storm), rhabdomyolysis, and acute renal and liver failure; responsible for permanent alterations or even death (Leon 2007). As mentioned above, despite hospital intervention, victims frequently die from multi-organ system failure during the weeks, months, and years following the heat exposure (Bouchama and Knochel 2002). For example, military heat stroke patients exhibit a ~2-fold increased mortality risk from cardiovascular, kidney, and liver failure within 30 years of hospitalization compared with patients admitted for a non-heat related illness (Leon and Helwig 2010a). Understanding the pathophysiology of heat-related illnesses is imperative in order to accurately predict the potential outcomes of heat exposure and develop specific treatment and mitigation protocols.

Insulin Metabolism

Insulin is the primary acute anabolic endocrine signal and has a critical role in carbohydrate, lipid and protein metabolism. Insulin increases glucose uptake, stimulates glycolysis, and promotes the synthesis of hepatic and muscle glycogen, adipose triglycerides and skeletal muscle protein; while simultaneously preventing their degradation. Although it is well described how insulin homeostasis is altered during different physiological and pathological conditions, the molecular mechanisms as well as the biological reasons behind such changes are frequently poorly understood. Further, there is increasing evidence that supports nutrients' role as signaling molecules rather than mere energetic substrates (Blad *et al.* 2011), which

accentuates the importance of understanding the triggers and consequences of changes in insulin homeostasis (the main coordinator of nutrient partitioning) that take place during specific conditions.

Insulin synthesis

Insulin is solely synthesized by β cells present in the pancreatic islets of Langerhans where they are co-located with α and δ cells, responsible for glucagon and somatostatin secretion, respectively. The insulin gene (*INS*) encodes a single chain prohormone: preproinsulin (Hadley 2000). During translation, a signal sequence of this immature hormone is cleaved in the endoplasmic reticulum resulting in the proinsulin molecule, which is then transported to the Golgi as part of the secretory pathway (Hadley 2000). Once in the secretory granules the connecting peptide (C-peptide) is excised from the precursor resulting in the mature insulin molecule composed of chain A and B joined by disulfide bonds (Hadley 2000). Insulin synthesis is controlled both at the transcriptional and translational level, with glucose being the main stimulus for its gene expression, transcript stability and rate of translation (Fu *et al.* 2013).

Insulin secretion

When plasma glucose rises, β cells' glucose uptake increases mainly via GLUT-2. Intracellularly, glucose is metabolized resulting in an increase in the ATP/ADP ratio, and the closure of ATP-sensitive potassium channels (K_{ATP} channels). As a result, the cell membrane depolarizes, leading to the opening of voltage dependent calcium channels and the influx of extracellular calcium. The elevation of intracellular calcium triggers the exocytosis of insulin (and C-peptide) granules to the portal vein (Prentki and Matschinsky 1987).

Although species dependent, glucose stimulated insulin secretion (GSIS) at supraphysiological levels (e.g. during a glucose tolerance test) is biphasic, with a first phase or

triggering phase lasting 5 – 6 minutes, which represents the secretion of the readily releasable pool of insulin granules; and a second phase or amplification phase lasting over 60 minutes, which represents the replenishment of the readily releasable pool by the reserve pool of insulin granules (Komatsu *et al.* 2013). However, the electrophysiological events (i.e. K_{ATP} channel closure and intracellular calcium elevation) are not biphasic suggesting that there are K_{ATP} channel independent mechanisms that modulate insulin secretion (Grill and Björklund 2000; Komatsu *et al.* 2013).

Insulin secretagogues

Even though glucose is the most potent stimulator of insulin secretion, other nutrients are capable of triggering insulin release and more importantly augment or amplify GSIS. Certain combinations of amino acids (e.g. glutamine and leucine) are oxidized in β cells which generates ATP and increases GSIS (Yang *et al.* 2010; Newsholme *et al.* 2014). Fatty acids can also enhance GSIS by intracellular oxidation, but also through interacting with G protein-coupled membrane receptors (e.g. GPR40; Morgan and Dhayal 2009; Vinolo *et al.* 2012; Newsholme *et al.* 2014). Interestingly, exposure to high concentrations of glucose and/or fatty acids have gluco- and lipo-toxic effects on β cells, with detrimental consequences on cellular function and viability (Grill and Björklund 2000). In addition, dietary nutrients can indirectly increase insulin release by stimulating incretin secretion. Incretins (glucagon-like peptide-1, GLP-1; and gastric inhibitory peptide) are intestinal hormones with insulinotropic activity, secreted in response to food ingestion, integrating diet chemical composition into the regulatory mechanism of insulin release (Grill and Björklund 2000; Gribble 2012). The relative importance of incretins on insulin secretion is illustrated by the fact that an oral glucose dose triggers a greater insulin response compared to an equivalent intravenous dose (Unger *et al.* 1968).

Another hormone capable of modulating insulin secretion is prolactin. Prolactin is required for proper islet development, as demonstrated by the decrease in β cell mass experienced by pancreatic prolactin receptor deficient mice (Ben-Jonathan *et al.* 2006). Prolactin stimulates β cell proliferation (Brelje and Sorenson 1991) and decreases the threshold for GSIS (Ramos-Roman 2011; Carre and Binart 2014), which makes it crucial for adapting to the increasing insulin resistance that homeorhetically occurs during pregnancy (Hughes and Huang 2011). The mechanism by which prolactin enhances insulin secretion appears mediated by the increase in glucose uptake and utilization by β cells, as it increases GLUT-2 mRNA and glucokinase activity (Weinhaus *et al.* 2007). Further, prolactin stimulates pyruvate dehydrogenase (PDH) activity probably by decreasing its inhibitor pyruvate dehydrogenase kinase (PDK) mRNA (Arumugam *et al.* 2010) and thus ensures complete glucose oxidative phosphorylation by enhancing flux through the tricarboxylic acid (TCA) cycle. Prolactin's insulinotropic properties are perplexing as it typically facilitates lactation (although species dependent; Lacasse *et al.* 2012), while increased systemic insulin action is associated with metabolic adaptions that prevent maximum milk yield (Bauman and Currie 1980). Identifying prolactin's role in modifying insulin's nutrient partitioning responsibilities is of obvious interest prior to and following parturition.

Counterintuitively, bacterial components might influence insulin secretion. *In vivo* lipopolysaccharide (LPS) infusion acutely (< 1 h) increases circulating insulin in both lactating and growing ruminants (Waldron *et al.* 2006; Rhoads *et al.* 2009b; Burdick Sanchez *et al.* 2013) and growing pigs (Baumgard et al., unpublished), which is paradoxical as endotoxemia is a potent catabolic condition accompanied by severe pyrexia and marked hypophagia. This increase in circulating insulin may be partially explained by the LPS-induced increase in skeletal

muscle insulin resistance, with the ostensible objective to divert nutrients toward the liver and the immune system, in order to mount an acute phase and inflammatory responses (Kimball *et al.* 2003; Orellana *et al.* 2008; Liang *et al.* 2013). Although inconsistent (Hagiwara *et al.* 2009), similar results are observed *in vitro* (Vives-Pi *et al.* 2003; Bhat *et al.* 2014), suggesting a direct effect of LPS on the pancreas. The mechanism by which LPS increases insulin secretion is unknown but might be mediated by the transcription factor hypoxia-inducible factor 1-alpha (HIF1a). In immune cells, LPS stabilizes HIF1a even under normoxic conditions (Kuschel *et al.* 2012). If the effects of LPS on HIF1a were demonstrated in β cells, it could explain LPS-induced increase in insulin secretion as HIF1a stimulates glucokinase expression (Ochiai *et al.* 2011) and thus glucose utilization and GSIS within the β cells (Weinhaus *et al.* 2007). Moreover, LPS might indirectly increase insulin release by stimulating other insulinotropic signals like GLP-1 (Kahles *et al.* 2014). Determining how LPS alters insulin secretion dynamics independent of changes in circulating glucose would provide important clues as the mechanisms, and maybe the rationale, for why LPS appears to partially regulate insulin secretion. Regardless, insulin secretion results from the integration of multiple signals in addition to hyperglycemia.

Insulin Signaling

The insulin receptor is a transmembrane dimer that belongs to the tyrosine kinase superfamily. Each monomer is the product of the insulin receptor gene (*INSR*), which after transcription undergoes alternative splicing to produce two isoforms: IR-A, and IR-B (the most abundant in muscle, adipose tissue, and liver). Post-translationally, the single chain protein undergoes proteolytic cleavage resulting in the α and β subunits that remain bound by intra-monomer disulfide bonds (Hadley 2000). The α subunit is extracellular, while the β subunit is comprised of extracellular, transmembrane and intracellular regions. The latter contains the

tyrosine kinase domain and regulatory regions. The two monomers are bound to each other by disulfide bonds between α subunits resulting in the mature insulin receptor, which is present in the cell membrane as a pre-dimerized receptor (Hadley 2000).

Upon insulin binding, the receptor is activated and undergoes autophosphorylation, which initiates a signaling cascade. Two canonical signaling pathways are well established: the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, responsible for insulin's metabolic effects; and the Ras/MAP kinase pathway, accountable for insulin's effects on cell growth and proliferation (Taha and Klip 1999). In the PI3K/Akt pathway, the activated receptor phosphorylates tyrosine residues of insulin receptor substrate (IRS; a surrogate protein for particle assembly), creating binding sites for the Src homology 2 (SH2) domain of phosphatidylinositol 3-kinase (PI3K). The active (phosphorylated) PI3K is translocated to the membrane where it catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). The latter activates PIP3-dependent protein kinase (PDK) which in turn activates protein kinase B or Akt by phosphorylation of serine and threonine residues (Taha and Klip 1999). Activated Akt leads to the phosphorylation of multiple substrates that ultimately result in the metabolic effects of insulin, including glucose uptake, via activation of AS160 and subsequent translocation of glucose transporter 4 to the plasma membrane; glycogen synthesis by inactivating glycogen synthase kinase-3; protein synthesis by regulation of the mammalian target of rapamycin (mTOR); lipolysis inhibition by activating phosphodiesterase and cyclic AMP (cAMP) hydrolysis, etc.

Integrative Metabolism

In the well-fed and post-prandial states, glucose, amino acids, and triglycerides are plentiful in blood and these dietary nutrients stimulate insulin secretion, which activates anabolic pathways. At the skeletal muscle, insulin decreases endothelial lipoprotein lipase (LPL) activity preventing dietary triglyceride hydrolysis, NEFA uptake, and thus NEFA utilization by muscle cells. Further, insulin-induced glucose uptake decreases NEFA utilization by preventing their access to the mitochondrial matrix for β oxidation (Berg *et al.* 2002). Briefly, increased intracellular glucose concentration and oxidation result in excess acetyl CoA accumulation and citrate leakage from the tricarboxylic acid (TCA) cycle. Citrate (allosterically) and insulin-induced phosphatases (covalently) stimulate acetyl CoA carboxylase (ACC), which catalyzes the transformation of acetyl CoA into malonyl CoA. Ultimately, malonyl CoA inhibits carnitine palmitoyltransferase 1 (CPT-1), blocking the translocation of NEFA into the mitochondria (Berg *et al.* 2002). As cellular energetic demands are met, insulin stimulates glycogen synthesis by activating kinases that lead to the phosphorylation (inhibition) of glycogen synthase kinase-3 and activation of glycogen synthase. Reciprocally, increased intracellular glucose-6-phosphate and ATP levels prevent glycogen degradation by inhibiting glycogen phosphorylase (Berg *et al.* 2002). Skeletal muscle protein synthesis is also enhanced by activation of the mTOR pathway by insulin and nutrients (mainly leucine; Berg *et al.* 2002). In adipose tissue, insulin activates LPL increasing the access of dietary NEFA to the adipocytes for storage. The location of *de novo* fatty acid synthesis varies among species. In swine, lipogenesis takes place in the adipose tissue where insulin activates phosphodiesterases (PDE) that hydrolyze cAMP, and inactivates protein kinase A (PKA) and hormone sensitive lipase (HSL), among others; ultimately inhibiting adipose tissue mobilization (Berg *et al.* 2002). Similar to the skeletal muscle, insulin activates

adipose ACC and results in the generation of malonyl CoA. Then, fatty acids (typically palmitate) are synthetized from malonyl CoA, additional acetyl CoA, and NADPH through a series of reactions catalyzed by the fatty acid synthase complex. Fatty acids are stored in the form of triglycerides within the lipid droplet of adipocytes (Berg *et al.* 2002). In the liver (which has a critical role in maintaining blood glucose levels), insulin stimulates glycogen synthesis by similar mechanisms as in the skeletal muscle, and inhibits glycogen degradation, since increased intracellular glucose both directly and indirectly (via protein phosphatase 1, PP1) inactivates glycogen phosphorylase (Berg *et al.* 2002).

In the post-absorptive state, blood glucose concentration declines, which results in decreased insulin and enhanced glucagon secretion. Glucagon exerts its effects mainly at the liver by binding a G protein-coupled receptor and activating the synthesis of cAMP from ATP. Thus, glucagon increases hepatic glycogen degradation and glucose export into blood by stimulating and inhibiting glycogen phosphorylase and glycogen synthase, respectively, via cAMP and PKA activation (Berg *et al.* 2002). Moreover, the reduction in insulin-induced glucose uptake by peripheral tissues contributes to increasing blood glucose levels. As glycogen stores become depleted, hepatic fructose 2,6-bisphosphate levels which mirror the blood glucose concentration decrease, up-regulating gluconeogenesis or the synthesis of glucose from glycerol (from triglyceride lipolysis), lactate (the Cori cycle), and amino acids (protein mobilization;(Berg *et al.* 2002). At the adipose tissue, the lack of insulin and increased glucagon stimulation promote lipolysis via cAMP synthesis, and PKA and HSL activation (among others); and the export of NEFA and glycerol to blood. As fasting progresses, glucose is spared for tissues that are obligate glucose utilizers (brain, red blood cells) while other fuels are recruited for metabolically flexible tissues (Berg *et al.* 2002). The liver and skeletal muscle shift from

glucose to NEFA utilization, as increased intracellular palmitoyl concentrations and AMP-activated protein kinase (AMPK) activation (due to decreased cellular energetic status) allosterically inhibit ACC. ACC inhibition decreases malonyl CoA synthesis, activating CPT-1 and allowing the translocation of fatty acids into the mitochondria for β-oxidation (Berg *et al.* 2002). At the skeletal muscle, decreased insulin signaling and increased circulating glucagon stimulate protein breakdown in order to provide amino acids as hepatic gluconeogenic precursors via the Cori cycle. However, as starvation progresses, the use of amino acids is deemphasized in order to preserve muscle mass, and ketone bodies become the main energy source. Hepatic ketone body synthesis and export increase is the result of enhanced NEFA oxidation and oxaloacetate depletion (used as a gluconeogenic precursor), and ultimately acetyl CoA accumulation (Berg *et al.* 2002).

Effects of Heat Stress on Metabolism

It is now clear that HS alters metabolism independently of nutrient intake. Environmental hyperthermia induces a well-documented decrease in feed intake (FI), presumably in an attempt to decrease metabolic heat production (West 2003), which was traditionally assumed to be solely responsible for the negative effects of HS on animal production (Fuquay 1981). However, experiments that included thermoneutral controls that were pair-fed (PFTN) to their HS counterparts demonstrated that in lactating dairy cows the decrease in FI can only explain 30-50% of the heat induced-milk yield reduction (Rhoads *et al.* 2009a; Wheelock *et al.* 2010). Similarly, reduced intake only accounts for up to 50% of the blunted growth of HS chickens (Geraert *et al.* 1996; Zuo *et al.* 2014). In contrast, HS growing pigs (Pearce *et al.* 2013a) do not lose as much body weight (BW) as PFTN controls. Thus, HS

has direct effects on performance and metabolism that are not mediated by the concomitant reduction in FI. Further, as discussed below, the effects of HS on productivity are likely mediated by changes in metabolism that would not be anticipated based on the animals' plane of nutrition (Baumgard and Rhoads 2013).

Protein metabolism

Phenotypic changes indicate that HS alters protein metabolism as it decreases lean tissue accretion in multiple species (Close *et al.* 1971; Lu *et al.* 2007). The mechanisms by which hyperthermia decreases protein accretion are not well understood, but previous reports indicate that HS inhibits DNA, RNA and protein synthesis (Henle and Leeper 1979; Streffer 1982). Further, HS increases plasma markers of muscle catabolism. Specifically, HS increases plasma urea nitrogen in heifers (Ronchi *et al.* 1999), cows (Shwartz *et al.* 2009), and pigs (Pearce *et al.* 2013a). A more accurate indicator of muscle catabolism is circulating 3-methylhistidine or creatine, both of which are increased in HS lactating cows (Schneider *et al.* 1988), poultry (Yunianto *et al.* 1997), rabbits (Marder *et al.* 1990), pigs (Pearce *et al.* 2013a), and exercising men (Febbraio 2001).

Lipid metabolism

Despite a substantial reduction in FI, an increase in carcass lipid retention has been described in chickens (Geraert *et al.* 1996), growing pigs (Christon 1988), and lactating sows (Renaudeau *et al.* 2014) reared under HS conditions. Increased adiposity potentially results from decreased adipose tissue mobilization, since we and others have demonstrated that HS decreases plasma NEFA concentrations in growing ruminants (Ronchi *et al.* 1999; O'Brien *et al.* 2010), lactating cows (Rhoads *et al.* 2009a; Wheelock *et al.* 2010; Baumgard *et al.* 2011), sheep (Sano *et al.* 1999), growing pigs (Pearce *et al.* 2013a), chickens (Geraert *et al.* 1996), rodents (Sanders

et al. 2009; Morera *et al.* 2012), and exercising humans (Febbraio 2001); and HS cows have a blunted response to an epinephrine challenge (Baumgard *et al.* 2011). Mechanistically, HS reduces lipolytic and increases lipogenic enzyme activity in rodents (Torlinska *et al.* 1987) and growing pigs (Pearce *et al.* 2011), respectively. Further, adipose LPL gene expression is increased during HS compared to thermoneutral (TN) *ad libitum* conditions, which suggests an increase capacity to store dietary and hepatic-derived triglycerides (Sanders *et al.* 2009). Overall, the lack of adipose tissue mobilization is a paradox, as HS not only reduces FI, but also induces a well-described stress response with increased released of lipolytic signals (e.g. circulating cortisol and epinephrine; Beede and Collier 1986).

Carbohydrate metabolism

The effects of HS on blood glucose are conflictive as there is evidence for it to increase (Febbraio 2001), decrease (Geraert *et al.* 1996; O'Brien *et al.* 2010; Baumgard *et al.* 2011) or remain unchanged (Sano *et al.* 1999; Rhoads *et al.* 2009a; Wheelock *et al.* 2010; Pearce *et al.* 2013a). Reasons for these discrepancies might reside in differences in species, physiological status, experimental design and heat protocols, etc. Regardless of basal plasma glucose, HS appears to alter carbohydrate metabolism. For instance, humans exercising in heat have increased blood glucose probably due to increased glucose hepatic output coupled with decreased peripheral glucose uptake (Fink *et al.* 1975; Febbraio 2001). Further, ingesting carbohydrates is unable to inhibit the increase in hepatic glucose export under such conditions (Angus *et al.* 2001). Despite a decrease in glucose uptake by skeletal muscle, various reports observe an increase in glucose utilization (glycogen oxidation) at the expense of NEFA oxidation (Fink *et al.* 1975; Febbraio 2001) in humans exercising in the heat. In addition, HS increases the respiratory quotient, which suggests enhanced glucose oxidation (Hargreaves *et al.* 1996).

Studies under resting conditions are scarcer, but Dickson and Calderwood (1979) observed an increase in glucose metabolism induced by whole body hyperthermia. Similarly, HS dairy cows seem to increase glucose utilization as, despite a reduction in milk yield (and thus a decrease in glucose output), whole-body glucose production did not differ compared to PFTN counterparts (Baumgard *et al.* 2011). The increased reliance on glucose as a substrate might explain the increase in intestinal (Garriga *et al.* 2006; Pearce *et al.* 2013b) and renal (Ikari *et al.* 2005) glucose absorption observed during HS. It might also be the reason why during HS hepatic glycogenolysis, which has a central role in maintaining blood glucose levels, remains sensitive to adrenergic signals while adipose lipolysis does not (Baumgard *et al.* 2011).

Apart from increasing whole-body glucose utilization, HS appears to markedly alter cellular energetics. Specifically, HS seems to up-regulate aerobic glycolysis and de-emphasize oxidative phosphorylation for ATP production (Baumgard and Rhoads 2013), differential metabolism which resembles the Warburg effect utilized by cancerous cells (Warburg 1956; Kim and Dang 2006). In agreement, circulating lactate increases in humans exercising in the heat (Fink *et al.* 1975; Angus *et al.* 2001), HS growing steers (Elsasser *et al.* 2009), porcine malignant hyperthermia (Hall *et al.* 1980), and heated melanoma cells (Streffer 1988). Due to its shear mass, circulating lactate presumably stems from skeletal muscle in spite of normoxic conditions (Baumgard and Rhoads 2013). Further, lactate might be partially responsible for the heat-induced phenotypic changes as it mediates insulin's antilipolytic effects by binding to the adipocyte G protein-coupled receptor GPR81 (Ahmed *et al.* 2010).

Table 1. The effects of heat stress on plasma parameters in various species[a]

Metabolite	Species	Response	Reference
BUN[b]	Chickens (uric acid)	= / ↑	25 / 48
	Cows	↓ / ↑	45 / 1, 21, 32, 33, 38, 41, 43, 44, 47
	Humans	↑	15, 19
	Pigs	↑	34
	Rats	↑	12
	Rabbits	↑	27
	Sheep	=	5
NEFA[c]	Chickens	↓ / ↓↑	17 / 6
	Cows	↓ / = / ↑	1, 20, 37, 38, 39, 44, 47 / 7 / 32, 45
	Pigs	↓	18, 29, 34
	Rats	↓	9, 13, 14, 31
	Sheep	↓ / ↑	39 / 40, 42
Glucose	Cats	↓	1, 24
	Chickens	↓ / = / ↑	14, 17, 50 / 25, 36 / 16
	Cows	↓	1, 7, 8, 20, 32, 37, 38, 41, 43, 44, 47
	Dogs	↓	22
	Humans	↑	3, 4, 10, 11, 30
	Pigs	↑	18, 34, 35
	Rabbits	↑	27
	Rats	↓ / ↑	28, 29, 31 / 14
	Sheep	↓ / = / ↑	2, 26 / 32, 40 / 5
Insulin	Cows	= / ↑	7 / 33, 47
	Humans	=	23
	Pigs	↑	18, 34
	Rats	↑	31, 46
	Sheep	↑	26, 40

[a]Adapted from: (Sanders 2010; Johnson 2014; Pearce 2014)
[b]Blood urea nitrogen
[c]Non-esterified fatty acids

[1](Abeni *et al.* 2007)
[2](Achmadi *et al.* 1993)
[3](Al-Harthi *et al.* 1990)
[4](Angus *et al.* 2001)
[5](Bell *et al.* 1989)
[6](Bobek *et al.* 1997)
[7](Burdick Sanchez *et al.* 2013)
[8](Burge *et al.* 1951)
[9](Burger *et al.* 1972)
[10](Febbraio *et al.* 1994)
[11](Febbraio 2001)
[12](Francesconi and Hubbard 1986)
[13](Frankel 1968)
[14](Frascella *et al.* 1977)
[15](Fukumoto *et al.* 1988)
[16](Garriga *et al.* 2006)
[17](Geraert *et al.* 1996)
[18](Hall *et al.* 1980)
[19](Hart *et al.* 1980)
[20](Itoh *et al.* 1998)
[21](Kamiya *et al.* 2006)
[22](Kanter 1954)
[23](Kappel *et al.* 1997)
[24](Lee and Scott 1916)
[25](Lin *et al.* 2006)
[26](Mahjoubi *et al.* 2014)
[27](Marder *et al.* 1990)
[28](Miova *et al.* 2013)
[29](Mitev *et al.* 2005)
[30](Monteleone and Keefe 1969)
[31](Morera *et al.* 2012)
[32](Nardone *et al.* 1997)
[33](O'Brien *et al.* 2010)
[34](Pearce *et al.* 2013a)
[35](Prunier *et al.* 1997)
[36](Rahimi 2005)
[37](Rhoads *et al.* 2009a)
[38](Ronchi *et al.* 1999)
[39](Sano *et al.* 1983)
[40](Sano *et al.* 1999)
[41](Settivari *et al.* 2007)
[42](Sevi *et al.* 2002)
[43](Shehab-El-Deen *et al.* 2010)
[44](Shwartz *et al.* 2009)
[45](Srikandakumar and Johnson 2004)
[46](Torlinska *et al.* 1987)
[47](Wheelock *et al.* 2010)
[48](Yalcin *et al.* 2009)

Pyruvate dehydrogenase kinases inactivate the PDH complex, preventing pyruvate's flux through the TCA cycle and oxidative phosphorylation, presumably forcing it towards either lactate or alanine synthesis (Berg *et al.* 2002). However, in the above mentioned studies, HS animals were compared to TN *ad libitum* controls and feed restriction by itself is known to increase PDK4 expression (Furuyama *et al.* 2003). Thus, further research is required in order to elucidate the molecular mechanisms as well as the biological reasons behind the changes observed in cellular energetics during HS.

Insulin homeostasis

Reasons why, despite reduced FI and increased circulating stress hormones, HS animals fail to mobilize AT and increasingly rely on glucose as an energetic substrate might be related to changes in insulin homeostasis. Heat stress increases circulating insulin in steers (O'Brien *et al.* 2010), lactating cows (Wheelock *et al.* 2010), sheep (Sano *et al.* 1999; Mahjoubi *et al.* 2014), pigs (Pearce *et al.* 2013a), and rodents (Morera *et al.* 2012) relative to PFTN controls. Even when compared to TN *ad libitum* controls and considering FI differences, plasma insulin has been reported to be elevated in HS heifers (Burdick Sanchez *et al.* 2013), lactating cows (Itoh *et al.* 1998), and sheep (Achmadi *et al.* 1993). Similarly, Tao and colleagues (2012) observed an increase in plasma insulin in HS lactating cows relative to cooled controls. Interestingly, exposing β cells to HS *in vitro* decreases insulin secretion (Kondo *et al.* 2012), which suggests that the heat-induced circulating insulin response observed *in vivo* might be triggered by secondary signals rather than direct pancreatic hyperthermia. As discussed in the insulin secretagogues section, both prolactin and LPS increase insulin secretion and might play a role in the metabolic changes observed during HS. For instance, plasma prolactin is elevated in response to HS in a variety of species and models (Alamer 2011; Wright *et al.* 2012).

Remarkably, prolactin concentration increases more than 3 fold after 30 minutes of heat exposure in humans (Iguchi *et al.* 2012). Prolactin's role in the adaptation to heat is not fully understood, but might be involved in water homeostasis and the sweating response (Collier *et al.* 1982; Kaufman and Mackay 1983), and in pelage molting (Foitzik *et al.* 2009). In addition, prolactin might participate in the cellular protection mechanisms as it increases heat shock proteins (HSP) in several tissues (Swindell *et al.* 2009). Further, prolactin inhibits lipolysis in adipose tissue explants (LaPensee *et al.* 2006; Brandebourg *et al.* 2007), which might point to its involvement in the lack of adipose tissue mobilization observed during HS. Circulating LPS also affects insulin secretion and is increased during HS (Hall *et al.* 2001; Pearce *et al.* 2013c), presumably due to hyperthermia's deleterious effect on intestinal barrier function and the subsequent increase in intestinal permeability to luminal content (Lambert *et al.* 2002), as further discussed later in this review.

Nonetheless, circulating insulin is the result of the interplay between insulin secretion and peripheral insulin signaling. The effects of HS on farm animals' whole-body insulin sensitivity have not been extensively studied and remain unclear (see literature summary on Table 2).

A consistent finding is an increase in the insulin response to a glucose tolerance test during HS. However, when considering the corresponding glucose disposal, there is evidence for insulin sensitivity to be either increased (i.e. glucose disposal is increased) or decreased (i.e. glucose disposal is decreased or unchanged).

Contradictory results are also observed in response to insulin tolerance tests (ITT) and hyperinsulinemic euglycemic clamps (HEC). These conflicting results might be due to differences in species, physiological status, experimental design and heat protocol, etc. For instance, to our knowledge we are the only ones who have performed a HEC in HS animals (i.e.

lactating cows) utilizing PFTN controls (Skrzypek *et al.* 2010), in order to elucidate the direct effects of HS. During a HEC, hyperinsulinemia is induced by infusing insulin at a constant rate. At the same time pre-hyperinsulinemia blood glucose levels are maintained (euglycemia) by infusing glucose and adjusting its rate (Muniyappa *et al.* 2008). Inducing hyperinsulinemia presumably inhibits both pancreatic insulin secretion and hepatic glucose output, and therefore, during the clamped state it is assumed that the amount of exogenous glucose entering the system equals the amount of insulin-induced glucose uptake by peripheral tissues. This is why the HEC is considered the gold standard technique to determine insulin sensitivity, as a greater rate of glucose infusion (ROGI) can be interpreted as an increase in peripheral glucose uptake or increased insulin sensitivity (Muniyappa *et al.* 2008).

Table 2. Effects of heat stress on insulin sensitivity

		GTT[a]		ITT[b]		HEC[c]	
Ref	Species	Glucose clearance	Insulin response	Glucose clearance	NEFA[d] reduction	ROGI[e]	Comments
1	Rodents	↑					Compared to PFTN[f]
2	Calves	=	↑	=			Compared to PFTN
3	Lactating cows	=	↑				Compared to TN *ad libitum*
4	Lactating cows	↑	↑				Compared to PFTN
5	Lactating cows	↓	↑				Compared to PFTN
6	Lactating cows	↑	=	↑	↓		Compared to cooled cows
7	Lactating cows			=		= (↑)	Compared to PFTN*
8	Sheep					=	Compared to TN *ad libitum*
9	Sheep					↓	Compared to TN *ad libitum*

[a]Glucose tolerance test; [b]Insulin tolerance test; [c]Hyperinsulinemic euglycemic clamp; [d]Non-esterified fatty acids; [e]Rate of glucose infusion; [f]Pair-fed thermoneutral; *When normalized to clamped plasma glucose

[1](Morera *et al.* 2012)
[2](O'Brien *et al.* 2010)
[3](Itoh *et al.* 1998)
[4](Wheelock *et al.* 2010)
[5](Baumgard *et al.* 2011)
[6](Tao *et al.* 2012)
[7](Skrzypek *et al.* 2010)
[8](Achmadi *et al.* 1993)
[9](Sano *et al.* 1999)

In the above mentioned study (Skrzypek *et al.* 2010), the ROGI did not differ between treatments; however, HS decreased baseline plasma glucose compared to PFTN conditions and therefore, HS animals required the same amount of glucose infused to maintain euglycemia at a lower level, indicating that HS lactating cows were more insulin sensitive than PFTN controls.

The increase in insulin sensitivity agrees with the human and rodent literature where thermal therapy (acute and short heat exposure repeated over time) improves insulin signaling in insulin resistance-related conditions like obesity (Gupte *et al.* 2009), diabetes (Hooper 1999; Kokura *et al.* 2007), and aging (Gupte *et al.* 2008; Gupte *et al.* 2011). Similar results are obtained by over-expressing HSP72 and utilizing HSP co-inducers (Chung *et al.* 2008), which indicates that the effects of heat treatment on insulin sensitivity are likely mediated by the up-regulation of HSP. In agreement, thermal therapy prevents c-jun amino terminal kinase (JNK) activation (a stress kinase responsible for the inactivation of IRS-1 and insulin resistance) in a HSP-dependent manner (Chung *et al.* 2008; Gupte *et al.* 2009; Gupte *et al.* 2011).

Not only does thermal therapy improve insulin sensitivity but, reciprocally, proper insulin action is also critical for survival and adaptation to a heat load. For example, diabetics are more susceptible to heat related-illness and death (Schuman 1972; Semenza *et al.* 1999) and insulin administration to diabetic rodents improves survivability to severe HS (Niu *et al.* 2003). This might be due to insulin's key role in mounting a HSP response and might explain why diabetics have decreased HSP72 expression, correlated with their degree of insulin resistance (Li *et al.* 2006).

The effects of HS on metabolism are highly conserved across species. Further, despite differences in their nature, both environmental hyperthermia and thermal therapy elicit similar responses on whole-body glucose utilization. In summary, the similarities among species and

models suggest that the changes in insulin homeostasis are well conserved, highlighting their importance in the adaptation to a thermal load.

Heat Stress and Intestinal Barrier Function

Heat stress increases intestinal permeability as reported in pigs (Pearce *et al.* 2013c), rodents (Hall *et al.* 2001; Lambert *et al.* 2002; Prosser *et al.* 2004), and humans (Lambert, 2004). In ruminants, HS causes rumen acidosis (Kadzere *et al.* 2002), which by itself can compromise intestinal barrier function (Plaizier *et al.* 2008). Further, HS markedly alters intestinal morphology, shortening the villi and causing epithelial sloughing (Yu *et al.* 2010; Pearce *et al.* 2013c), which might affect digestion and absorption of nutrients (Liu *et al.* 2009), and ultimately compromise animal performance. The effects of HS on intestinal permeability are likely a consequence of heat dissipation mechanisms. During HS, blood flow is diverted from the viscera to the skin in an attempt to maximize radiant heat dissipation (Kregel *et al.* 1988). As a compensatory mechanism to maintain whole-body blood pressure, the visceral vasculature vasoconstricts (Lambert 2009), decreasing blood flow and ultimately depriving the splanchnic tissues from oxygen and nutrients (McGuire *et al.* 1989; Hall *et al.* 1999). As demonstrated by their early upregulation of HSP during hyperthermia (Flanagan *et al.* 1995), enterocytes are extremely sensitive to oxygen and nutrient restriction (Rollwagen *et al.* 2006). The combination of hyperthermia, and oxygen and nutrient deprivation results in hypoxia, ATP depletion, and oxidative and/or nitrosative stress in the enterocyte (Hall *et al.* 2001; Yan *et al.* 2006), and subsequent cell damage and tight junction disruption (Lambert 2004). Moreover, the intestinal epithelium damage leads to local inflammation (Pearce *et al.* 2013c), which further enhances tight junction dysfunction (Al-Sadi and Ma 2007). Ultimately, HS compromises the intestinal

barrier function increasing the permeability to luminal content (e.g. LPS from bacterial origin), which in conjunction with a heat-induced impairment of the intestinal detoxification mechanisms (Pearce *et al.* 2013c), results in increased LPS appearance in portal and systemic circulation (Lambert 2004). In agreement, circulating LPS increases in HS pigs (Pearce *et al.* 2013c), chickens (Cronje 2007), rodents (Hall *et al.* 2001; Lim *et al.* 2007), monkeys (Gathiram *et al.* 1988), and human heat-stroke patients (Bouchama *et al.* 1991). Lipolysaccharide is a potent immunogenic signals and the subsequent inflammatory response might mediate some of the negative effects of HS. For instance, LPS plays a central role in heat stroke pathophysiology, as survival increases when intestinal bacterial load is reduced (Bynum *et al.* 1979; Gathiram *et al.* 1987b) or when plasma LPS is neutralized (Gathiram *et al.* 1987a). In conclusion, compromised barrier function's relative role on heat-induced effects on human health and animal productivity remains poorly understood and is of obvious academic and practical interest.

Similarities between heat stress and endotoxemia

Animals experiencing heat stroke or severe endotoxemia share many physiological and metabolic similarities (Lim *et al.* 2007). Phenotypically, both models induce lethargy and a voluntary decrease in appetite (Elsasser *et al.* 1995; West 2003). During endotoxemia, LPS interaction with toll-like receptor 4 (TLR4) on immune cells results in activation of the NFκB pathway, leading to cytokines synthesis and secretion (Lu *et al.* 2008). Similarly, heat-stroke patients typically suffer from a systemic inflammatory response syndrome presumably as a consequence of increased circulating cytokines (Leon and Helwig 2010b). In response to endotoxemia, skeletal muscle protein is mobilized and amino acids are partitioned toward the liver and immune system to provide amino acids as substrate for the synthesis of acute phase response proteins and cytokines (Kimball *et al.* 2003; Orellana *et al.* 2008). Likewise, an

increase in plasma markers of muscle degradation has been described during HS (Baumgard and Rhoads 2013). Moreover, both models appear to increase circulating insulin (Vives-Pi *et al.* 2003; Waldron *et al.* 2006; Baumgard and Rhoads 2013; Burdick Sanchez *et al.* 2013) and glucose utilization at the expense of NEFA (Frisard *et al.* 2010; Baumgard and Rhoads 2013). Collectively, the multiple metabolic similarities between HS and endotoxemia point to LPS having a key role in the pathophysiology of heat-related illnesses.

Summary

Heat stress is a major environmental hazard for both humans and animals. Despite the high morbidity and mortality of heat-related illnesses, their pathophysiology remains poorly understood, which prevents the development of targeted treatment protocols against specific aspects of these diseases. For animal agriculture, HS is a welfare and economic issue that jeopardizes the efficient production of high quality animal protein. Further, climatic models predict sustained global warming and increased frequency of extreme weather events like heat waves, which makes it imperative to define the biology and mechanisms of how HS risks human health and animal performance.

Across species, changes in metabolism appear critical to adapt to and ultimately survive a heat load. Specifically, altered insulin homeostasis might play a major role in the phenotypic changes observed in animals reared under environmentally-induced hyperthermia. Moreover, an increase in insulin action might facilitate the increase in glucose utilization observed during HS. Thus, this dissertation's objectives were to determine the effects of HS on basal and stimulated metabolism (Chapter 2) and insulin homeostasis (Chapter 3).

In addition, metabolic similarities between models of HS and endotoxemia indicate that the heat-induced intestinal barrier dysfunction and the subsequent increase in luminal pro-inflammatory molecules permeability might partially mediate the deleterious effects of HS on health and productivity. In animal agriculture, nutritional management constitutes an easily adjustable and readily implementable tactic that could be utilized in order to mitigate the effects of HS on intestinal barrier function and potentially in productivity. Thus, this dissertation's last objective was to evaluate the feasibility of dietary intervention as a tool to alleviate the effects of HS on intestinal health (Chapters 4 and 5).

CHAPTER 2: EFFECTS OF HEAT STRESS ON BASAL AND STIMULATED METABOLISM

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- M.V.S.F. and L.H.B. designed the experiment, interpreted the data, drafted the manuscript, and prepared the figures.
- M.V.S.F., J.S.J., M.A., S.K.S. and L.H.B. conducted the live phase of the experiment.
- J.T.S., J.W.R., L.C. and S.C.I. performed and helped to interpret the gene expression data.
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- R.P.R. provided intellectual contributions key to the success of the experiment.
- All authors contributed to revision of the manuscript and approved this version.

Abstract

Heat stress (HS) jeopardizes human and animal health and reduces animal agriculture productivity; however, its pathophysiology is not well understood. Study objectives were to evaluate the effects of HS on basal and stimulated energetic metabolism. Female pigs (57±5 kg body weight) were subjected to two experimental periods. During period 1, all pigs remained in thermoneutral conditions (TN; 20°C) and were *ad libitum* fed. During period 2, pigs were exposed to: 1) constant HS conditions (32°C) and fed *ad libitum* (n=7), or 2) TN conditions and pair-fed (PFTN; n=10) to eliminate the confounding effects of dissimilar feed intake. All pigs received an intravenous glucose tolerance test (GTT) and an epinephrine challenge (EC) on period 1, and during the early and late phases of period 2. After 8 d of environmental exposure, all pigs were sacrificed and tissue samples were collected. Despite a similar reduction in feed intake (39%), HS pigs tended to have decreased circulating non-esterified fatty acids (NEFA; 20%) and a blunted NEFA response (71%) to the EC compared to PFTN pigs. During early exposure, HS increased basal circulating C-peptide (55%) and decreased the insulinogenic index (45%) in response to the GTT. Heat-stressed pigs had a reduced T_3 to T_4 ratio (56%) and hepatic 5'-deiodinase activity (58%). After 8 days of environmental exposure, HS decreased the expression of genes involved in lipolysis in adipose tissue, and in oxidative phosphorylation in liver and skeletal muscle. In summary, HS markedly alters both lipid and carbohydrate metabolism independently of nutrient intake.

Keywords: Heat Stress, Basal Metabolism, Glucose Tolerance Test, Epinephrine Challenge, Thyroid Hormones

Introduction

Heat stress (HS) is a major environmental hazard for both humans and animals. Heat claims more human lives than all other climatic events combined (Changnon *et al.* 1996), with the young and elderly populations being the most susceptible (Leon and Helwig 2010a). Surprisingly, despite increased understanding on the pathophysiology of heat-related illnesses (Bouchama and Knochel 2002), the only standard procedures to treat heat victims are cooling and re-hydration (Leon and Helwig 2010a).

In addition to morbidity and mortality, HS negatively impacts livestock productivity. Environmental hyperthermia costs global animal agriculture several billion dollars annually due to reduced and inconsistent growth, decreased carcass quality, compromised reproduction, reduced milk yield and egg production, and increased veterinary costs (St-Pierre *et al.* 2003; Baumgard and Rhoads 2013). We have previously reported that, despite hypercatabolic hallmarks like marked hypophagia and weight loss, HS increases basal and stimulated circulating insulin and decreases adipose tissue mobilization in a variety of species (Baumgard and Rhoads 2013), including pigs (Pearce *et al.* 2013a). Interestingly, diabetic humans and rodents are more susceptible to heat-related illnesses and exogenous insulin rescues this phenotype (Semenza *et al.* 1999; Niu *et al.* 2003). Further, thermal therapy improves insulin sensitivity in diabetic and obese rodents (Kokura *et al.* 2007; Gupte *et al.* 2009) and humans (Hooper 1999). Collectively, these reports suggest that insulin or altered insulin action and subsequent shifts in metabolism are a conserved response among species that might play an important role in the adaptation and survivability to HS (Baumgard and Rhoads 2013).

Thus, the first study objective was to determine the effects of HS on the temporal responses to acute metabolic challenges in pigs. Our second objective was to investigate the

systemic and molecular changes responsible for the HS-induced shift in post-absorptive energetic metabolism. The pig is both a relevant biomedical model (Prather *et al.* 2013) and an important agricultural species, thus results will likely have important implications in both human health and animal production.

Materials and Methods

Animals and experimental design

Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Seventeen crossbred female pigs (57±5 kg body weight) were randomly assigned to 1 of 2 treatments during 2 experimental periods. During period (4 d in length), all pigs were exposed to thermoneutral conditions (TN; 20°C, ~35% humidity) and fed *ad libitum*. During period 2 (8 d in length), pigs were either exposed to constant HS conditions (32°C, ~23% humidity; n=7) and fed *ad libitum* or remained in TN conditions and were pair-fed (PFTN; n=10) to their HS counterparts, in order to eliminate the confounding effect of dissimilar nutrient intake. For the pair-feeding calculations, as-fed period 1 daily feed intake (FI) was averaged for each pig and used as a baseline. For each HS pig, the decrease in intake during period 2 was calculated as the percentage of FI reduction relative to period 1 for each day of HS exposure. This percentage of FI reduction was averaged for all the HS pigs per day of exposure and applied individually to the baseline of each PFTN pig. The calculated amount of feed was offered to the PFTN pigs three times a day (~0800, 1400, and 2100 h) in an attempt to minimize large post-prandial shifts in metabolism. All pigs were fed a standard swine grower diet consisting mainly of corn and soybean meal formulated to meet or exceed nutrient requirements (National Research Council 2012). Pigs were individually housed in metabolic crates in 1 of 6

environmental chambers where temperature was controlled but humidity was not governed. Both parameters were recorded every 30 min by a data logger (Lascar EL-USB-2-LCD, Erie, PA). Rectal temperature was measured with a digital thermometer (ReliOn, Waukega, IL), respiration rate was determined by counting flank movements, and both indices were measured twice a day (0700 and 2200 h) and condensed into daily averages. Pigs were sacrificed on d 8 of period 2 using the captive bolt technique and subcutaneous adipose tissue (AT) from the cranial dorsum, skeletal muscle from longissimus dorsi (LD), liver, and pancreas samples were immediately collected and snap frozen or preserved for histology.

Blood sampling and metabolic challenges

An indwelling jugular catheter was surgically inserted on d 1 of period 1 in all pigs. Pigs were anesthetized with a mixture of tiletamine/zolazepam (Telazol®, Fort Dodge Laboratories Inc., Fort Dodge, IA), ketamine (Ketaject®, Clipper Distributing Company, LLC, St Joseph, MO), and xylazine (Anased®, Lloyd Inc., Shenandoah, IA) for a final concentration of 100, 50, and 50 mg/ml, respectively. Pigs were injected IM with the anesthetic mixture at 1 ml/23 kg BW dose. With the pigs in dorsal recumbency, the jugular vein was located with a 14 G, 3.75 cm introducer needle (Mila International Inc., Erlanger, KY) using a percutaneous technique. Once in the vein, Tygon® tubing (1.016 mm inside diameter, 1.778 mm outside diameter; Saint-Gobain Performance Plastic Corp., Aurora, OH) was inserted through the introducer needle (15 cm). After removing the introducer, a small skin incision was made cranial to the tube's insertion point exposing the subcutaneous tissue. Using laparoscopic forceps (Auto Suture Endo Grasp^{TM} 5mm, Covidien, Mansfield, MA), the tube was tunneled subcutaneously from the incision and exteriorized at the dorsum of the back. The neck incision was sutured and the catheter remained subcutaneous and not exposed. All pigs received antibiotics (Ceftiofur,

Excede®, Pfizer Animal Health, New York, NY) and non-steroidal anti-inflammatory drugs (Flunixin meglumine, Banamine-S, Schering-Plough Animal Health Corp., Whitehouse Station, NJ) during the surgery. Intravenous antibiotic therapy continued daily for the rest of the study (Ampicillin, Polyflex®, Fort Dodge Laboratories Inc., Fort Dodge, IA) and catheters were flushed twice daily with heparinized saline (100 U/ml).

Animals were fasted for 2 hours prior to the metabolic challenges and daily blood sampling. Glucose tolerance tests (GTT) were performed on d 3 of period 1 and d 1 and 6 of period 2. A 50% dextrose solution bolus (VetOne®, MWI Veterinary Supply, Boise, ID) was administered at 0.5 g/kg BW dose via the jugular catheter. Blood samples were collected at -20, -10, 0, 5, 7.5, 10, 15, 20, 30, 45, 60 min relative to the glucose administration. Epinephrine challenges (EC; 3 μg/kg BW; American Regent, Inc., Shirley, NY) were performed on d 4 of period 1 and d 2 and 7 of period 2 and blood samples were collected at -20, -10, 0, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60 min relative to the epinephrine administration. Daily blood samples were obtained at 0800 h. All blood samples were collected into disposable glass tubes containing 250 U of sodium heparin and immediately placed on ice. Plasma was harvested by centrifugation at 1300 x *g* for 15 min at 4°C and stored at -80°C for later analysis.

Blood parameters analyses

Plasma glucose and non-esterified fatty acids (NEFA) concentrations were measured enzymatically using commercially available kits (Wako Chemicals USA, Richmond, VA). The intra- and inter-assay coefficients of variation were 4.3 and 3.6%, and 7.3 and 5.8% for glucose and NEFA, respectively. Plasma β-hydroxybutyrate (BHB) concentration was also analyzed enzymatically using a commercially available kit (Pointe Scientific Inc., Canton, MI) and the intra- and inter-assay coefficients of variation were 3.2 and 2.2%. Plasma insulin and C-peptide

concentrations were analyzed using ELISA kits (Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 2.9 and 6.3%, and 7.2 and 4.9% for insulin and C-peptide, respectively. Plasma lipopolysaccharide (LPS) binding protein (LBP) concentration was determined using an ELISA kit (Hycult® biotech, Uden, The Netherlands) and the intra-assay coefficients of variation was 1.5%. Total plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations were evaluated using commercially available solid phase RIA kits (MP Biomedicals, LLC; Irvine, CA) according to the manufacturer's instructions. The assay kits were validated (recovery and linearity of diluted samples) for use with porcine plasma samples as previously described (Kahl *et al.* 2000), and the intra-assay coefficients of variation were 3.7 and 4.1% for T_3 and T_4, respectively.

Hepatic 5'-deiodinase type I (5′D) activity

Outer-ring deiodinating activity was determined by quantifying the $^{125}I^-$ source released from 3,3′,5′-[^{125}I]-T_3 (rT_3) as previously described (Kahl *et al.* 2000). In brief, hepatic samples were homogenized in 0.01 M HEPES buffer (pH 7.0, 0.25 M sucrose, 5 mM EDTA). After centrifugation (30 min at 2000 × *g*), the supernatant was incubated for 5 min in 0.1 M phosphate buffer (pH 7.0, 1 mM EDTA) in the presence of 5 mM dithiothreitol at 37°C with approximately 80,000 cpm of [^{125}I]-rT_3 (DuPont-New England Nuclear, Boston, MA) and 500 nM of unlabeled rT_3 (Calbiochem, La Jolla, CA). The released $^{125}I^-$ source was isolated as trichloroacetic acid-soluble radioactivity. The 5′D activity was expressed as nmol of I^- produced per mg protein per hour. Protein concentration in homogenates was determined by bicinchoninic acid assay (BCA, Pierce™, Thermo Fisher Scientific Inc., Rockford, IL). The intra-assay coefficient of variation was 8.6%.

Pancreatic insulin content

Pancreatic protein was extracted at 4°C overnight with acid-ethanol (75% ethanol, 1.5% HCl). After centrifugation at 2000 rpm for 15 min, the supernatant was neutralized with 1M Tris (pH = 7.5) and total protein was determined by BCA assay (Pierce™, Thermo Fisher Scientific Inc., Rockford, IL). Neutralized samples were further diluted (1:20000) and insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions. Pancreatic insulin content was calculated relative to the total amount of protein.

Pancreatic immunohistochemistry

Pancreatic tissue samples were fixed in 10% formalin for 24 h and then transferred into 70% ethanol. Fixed samples were sectioned at 4 μm thickness. Two random sections per pig were stained for insulin using an indirect immunoperoxidase technique. The primary and secondary antibodies (Dako, Carpinteria, CA) were a polyclonal guinea pig anti-porcine insulin antibody (Dilution 1:1000) and a polyclonal rabbit anti-guinea pig horse radish peroxidase-conjugated immunoglobulin (Dilution 1:50), respectively. Diaminobenzidine (Dako, Carpinteria, CA) was used as the chromogen and Mayer's hematoxylin as the counterstain. Negative controls were generated by omitting the primary or the secondary antibody. Five non-overlapping fields per section were imaged at 50X using Q-capture Pro 6.0 software (Qimaging®, Surey, BC) with the operator being blind to the treatments. The relative stained area was measured by thresholding and the number and size of insulin stained cell clusters were determined using the particle analysis tool in ImageJ (US National Institutes of Health, Bethesda, MD). The results obtained in each section were averaged into a single measurement per pig.

RNA isolation and quantitative PCR

Total RNA was extracted from AT, LD and liver using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and was utilized for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Gene expression differences were determined using qPCR (BioMark^{TM} System, Fluidigm Corporation, San Francisco, CA) on 40, 44 and 44 genes for AT, LD and liver, respectively (Tables 5-7). Genes chosen for analysis were selected based on the RNA-Seq output of a similar experiment (Seibert *et al.* 2014). Expression normalization across samples within a tissue was done by calculating a delta Ct (ΔCt) value for each sample using *GAPDH* for AT, *β-actin* for LD and *RPL32* in liver, as transcript abundance for these genes proved to be the most similar between treatments for each given tissue ($P > 0.05$). Delta delta Ct (ΔΔCt) values were calculated utilizing a reference sample and fold differences between treatments were obtained by applying the equation $2^{\wedge|\Delta\Delta Ct\_PFTN-\Delta\Delta Ct\_HS|}$, where a positive and a negative value indicate an increase and a decrease in transcript abundance, respectively, in HS pigs relative to PFTN controls. Statistical analysis was performed on the ΔΔCt values and data is reported in the results section as relative fold difference.

Calculations and statistical analysis

Metabolic responses to the GTT and EC were calculated as area under the curve (AUC) by linear trapezoidal summation between successive pairs of metabolite concentrations and time coordinates after subtracting baseline values. Glucose and insulin AUC in response to the GTT were calculated through min 20 of the challenge. Glucose and NEFA responses to the EC were determine through min 30 and 15, respectively. For the GTT, glucose and insulin deltas were calculated for each challenge as the change in their concentration between 0 and 5 min, and 0

and 7.5 min, respectively. In addition, glucose disappearance was calculated as the slope of glucose concentrations between 5 and 30 min. An insulinogenic index was determined as the insulin AUC to glucose AUC ratio.

All data were statistically analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC). Single measurements were analyzed using PROC MIXED and the model included treatment as fixed effect. Variables with multiple measurements per pig over time were analyzed using PROC MIXED with day of period 2 as the repeated effect and period 1 values used as a covariate. Auto regressive and spatial power covariance structures were utilized for equally and unequally spaced measurements, respectively. The model included treatment, day and their interaction as fixed effects. For each variable, normal distribution of residuals was tested using PROC UNIVARIATE and logarithmic transformation was performed when necessary. Data are reported as least square means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

As expected, during period 2 HS pigs had increased rectal temperature and respiration rate (1.5°C and 4.5 fold, respectively; $P < 0.01$) compared to PFTN controls (Table 3). Heat stress decreased FI (39%, $P < 0.01$), and by design, PFTN pigs' FI was reduced similarly (Table 3).

During period 2, basal glucose concentrations decreased progressively with time ($P < 0.01$) in both treatments; however, HS pigs became overall more hypoglycemic (6%, $P = 0.02$) than PFTN pigs (Fig. 1A). No treatment differences were detected on basal plasma insulin (Fig. 1B) or the insulin to glucose ratio (Fig. 1C), but there was a treatment by day interaction on plasma C-peptide and C-peptide to glucose ratio ($P = 0.01$) as they were increased on d 1 (48 and

44%, respectively) and 3 (61 and 64%, respectively) in HS pigs compared to PFTN controls, but no treatment differences were detected on d 7 (Fig. 1D and E). During period 2, basal plasma NEFA acutely increased on d 1 and progressively decreased thereafter ($P < 0.01$) in both treatments, but overall HS pigs tended to have reduced basal NEFA (20%, $P = 0.07$) than PFTN pigs (Fig. 1F). There was a tendency for a treatment by day interaction on plasma BHB ($P = 0.10$), as HS pigs had increased BHB levels on d 1 (44%) compared to PFTN pigs, but similar BHB concentrations thereafter (Fig. 1G). Circulating LBP decreased with time ($P = 0.05$) and there was a tendency for a treatment by day interaction ($P = 0.08$), as HS pigs had numerically increased LBP concentrations on d 1 (20%) and d 3 (56%) compared with PFTN controls, but plasma LBP did not differ between treatments on d 7 (Fig. 1H). At the initiation of period 2, plasma T_3 concentrations decreased, but progressively increased over time ($P < 0.01$) for both treatments (Fig. 2A). However, the d 1 decrease was more severe in HS pigs (68%) and this difference was maintained through the end of period 2 ($P < 0.01$; Fig. 2A). There was a treatment by day interaction on plasma T_4 ($P < 0.01$) as HS pigs had decreased concentrations compared to PFTN pigs on d 1 and 3 (36 and 40%, respectively), but circulating T_4 did not differ between treatments on d 7 (Fig. 2B). Overall, HS pigs had decreased T_3 to T_4 ratio (56%, $P = 0.01$) compared to PFTN controls (Fig. 2C). After 8 d of environmental treatment, hepatic 5'D activity was reduced (58%; $P < 0.01$) in HS pigs compared to PFTN controls (Fig. 2D).

During period 2 there was a treatment by day interaction on the glucose response to the GTT ($P < 0.01$) as HS pigs had an increased glucose AUC (15%) on d 1, but not on d 6 (Table 4). There was a treatment by day interaction on the glucose delta in response to the GTT ($P = 0.04$), as it did not change from d 1 to 6 in PFTN pigs but decreased (10%) in HS pigs (Table 4). Glucose disappearance following glucose administration tended to increase (13%; $P = 0.06$)

from d 1 to 6, however overall, it tended to be decreased (14%; $P = 0.07$) in HS pigs compared to PFTN counterparts (Table 4). There was a tendency for a treatment by day interaction on the insulin response to the GTT ($P = 0.06$) as HS pigs had a decreased (30%) insulin AUC on d 1 compared to PFTN pigs, but the insulin response was similar between treatments on d 6 (Table 4). Overall, the insulin delta in response to the GTT tended to be reduced (16%, $P = 0.06$) in HS pigs compared to PFTN controls (Table 4). There was a treatment by day interaction on the insulinogenic index ($P < 0.01$) as it was decreased (45%) in HS pigs on d 1 compared to PFTN controls, but did not differ between treatments on d 6 (Table 4).

During period 2, the NEFA AUC in response to the EC decreased (43%; $P < 0.01$) from d 2 to 7, and overall it was reduced (71%; $P = 0.01$) in HS pigs compared to PFTN controls (Table 4). In contrast, the glucose response to the EC did not differ ($P > 0.10$) between treatments or change with time (Table 4).

After 8 d of environmental exposure, pancreatic insulin content did not differ ($P > 0.17$) between treatments (Fig. 3C). Based on the immunohistochemistry data, HS tended to decrease pancreatic insulin stained area (32%; $P = 0.08$) compared to PFTN conditions (Fig. 3A, B, D). In addition, there were no treatment differences detected in the quantity of insulin positive cell clusters (Fig. 3E). However, when classified by size, HS decreased (66%; $P < 0.01$) the percentage of larger clusters (diameter > 100 μm), while increasing (4%; $P \leq 0.01$) the percentage of smaller clusters (diameter < 50 μm; Fig. 3F).

After 8 d of environmental treatment, HS increased or tended to increase transcript abundance for heat shock proteins compared to PFTN conditions: *HSPA4* (2.43; $P = 0.08$), *HSPB8* (2.51; $P = 0.10$) and *HSPE1* (2.57; $P = 0.09$), in AT; *HSP90AA1* (2.10; $P < 0.01$), *HSPA4* (1.52; $P = 0.05$), *HSPCB* (1.72; $P = 0.02$) and *HSPE1* (2.38; $P = 0.02$), in LD; and

HSPE1 (1.62; *P* = 0.08), in liver (Tables 5-7). In AT, transcripts for the following genes were or tended to be less abundant in HS compared to PFTN pigs: *ATGL* (-1.56; *P* = 0.07), *INSR* (-1.46; *P* = 0.07), *MIF* (-1.60; *P* = 0.10), *PDAP1* (-1.73; *P* = 0.07), *PRKAG1* (-1.89; *P* = 0.05), and *ZSCAN29* (-1.77; *P* = 0.05; Table 5). In LD, HS conditions increased transcript abundance for *CD14* (1.68; *P* = 0.01), and reduced or tended to reduce it for *CKMT2* (-2.03; *P* = 0.10), *IDH2* (-1.77; *P* = 0.06), *NDUFS7* (-2.01; *P* = 0.06), *PDK4* (-2.53; *P* = 0.05), and *SLC16A5* (-2.60; *P* = 0.03); compared to PFTN conditions (Table 6). In liver, mRNA transcripts for *FASN* (2.62; *P* < 0.01), *KCTD6* (1.36; *P* = 0.08), and *RBM7* (1.31; *P* = 0.06) were or tended to be more abundant; while, *NDUFB7* (-1.28; *P* = 0.05) and *NDUFS7* (-1.29; *P* = 0.10) were or tended to be less abundant in HS pigs compared to PFTN controls (Table 7).

Discussion

Heat stress jeopardizes human health and compromises animal agriculture productivity. Despite advances in the understanding of heat-related illnesses, there is no treatment against specific aspects of their pathophysiology, and protocols are limited to generic cooling and hydration (Leon and Helwig 2010a). Similarly, improvements in barn design have failed to completely prevent the loss in animal productivity during the warm summer months (Stowell *et al.* 2009). Therefore, a better understanding of the biological consequences of HS is critical in order to develop treatment protocols and mitigation strategies.

Typically, during inadequate nutrient intake, AT is mobilized and NEFA contribution to whole-body oxidation is markedly increased (Vernon 1992). However in the current study, despite a 40% reduction in FI, HS pigs had reduced basal NEFA concentrations and this suggests that they did not mobilize as much AT as PFTN counterparts. Further, the NEFA response to the

EC was blunted in the HS pigs and this reduced sensitivity to lipolytic signals agrees with our ruminant data (Baumgard *et al.* 2011). The decrease in circulating markers of AT mobilization during HS is mechanistically supported by a reduction in both *ATGL* gene expression, a key enzyme of the lipolytic cascade (Zimmermann *et al.* 2004), as well as the gene encoding the AMPK regulatory subunit (*PRKAG1*). Interestingly, recent work has implicated AMPK in the regulation of ATGL content and lipase activity (Gaidhu *et al.* 2012), and it is tempting to speculate that their simultaneous reduction in transcript abundance is mechanistically related. The reduced capacity for AT to contribute to systemic energetics despite an increase in energy requirements during HS is consistent with research in agricultural animals and biomedical models (Torlinska *et al.* 1987; Shwartz *et al.* 2009; Pearce *et al.* 2013a). Interestingly, similar to our ruminant data (Baumgard *et al.* 2011), the glucose response to the EC, an indicator of hepatic glycogenolysis capacity, was similar between HS and PFTN pigs. These data indicate that AT becomes refractory to adrenergic signals, while the liver remains responsive during HS. The mechanism for the different responsiveness between the liver and AT is unknown, but a rationale for it could be the increased reliance on glucose as a whole-body fuel during HS as we and others have demonstrated (see review (Baumgard and Rhoads 2013). By remaining sensitive to catabolic signals, the liver can maintain its pivotal role as the glucose supplier to extra-hepatic tissues. Acute HS (24 h) increased plasma BHB, which is somewhat perplexing as circulating BHB is normally positively correlated with NEFA levels (Masoro 1977). However, this is not unprecedented as Ronchi et al. (1999) reported a similar discordant NEFA and BHB pattern in HS ruminants. The fact that circulating BHB increases without a concomitant increase in circulating NEFA might indicate that ketone body oxidation is decreased; thereby reducing their plasma clearance. Interestingly, HS increases circulating LPS (presumably from intestinal origin

as described later in this discussion) and we have recently demonstrated that LPS exposure reduces skeletal myoblast ketone oxidation (Rhoads and Baumgard, unpublished observation). Nonetheless, the mechanisms by which HS uncouples the relationship between circulating NEFA and BHB and its energetic consequences require further investigation.

Reasons why heat-stressed animals fail to mobilize AT despite being in a hypercatabolic condition might be related to changes in insulin homeostasis. Insulin is a potent antilipolytic signal and is frequently elevated in HS animals when compared with PFTN counterparts (Baumgard and Rhoads 2013). In contrast, we did not observe treatment differences in basal insulin; however C-peptide, a co-product of insulin's cleavage from proinsulin, was increased in HS pigs. C-peptide is thought to be a better indicator of pancreatic insulin secretion because it is produced at a 1:1 ratio to insulin and it avoids the confounding effects of hepatic insulin extraction (Wallace *et al.* 2004). Our observation of increased C-peptide suggests that HS-induced increase in circulating insulin is the result of increased pancreatic secretion rather than decreased systemic insulin clearance. Further, increased insulin secretion in heat-stressed pigs was associated with pancreatic insulin depletion as HS decreased pancreatic insulin content, ostensibly (i.e. insulin-stained area was reduced and pancreatic insulin protein content was numerically decreased) due to a reduction in insulin-positive cluster size rather than cluster number. Interestingly, insulin receptor (*INSR*) gene expression at the adipose tissue level was decreased in HS pigs, which is presumably an adaptive response to increased circulating insulin.

Exposing β cells to HS *in vitro* decreases insulin secretion (Kondo *et al.* 2012), which suggests that the heat-induced circulating insulin response observed *in vivo* might be triggered by secondary signals rather than direct pancreatic hyperthermia. One example may be prolactin, which increases in response to HS in a variety of species and models (Alamer 2011), including

humans (Iguchi *et al.* 2012) and our HS pig model (Sanz-Fernandez *et al.* 2012). Prolactin is thought to be involved in water homeostasis and the sweating response (Alamer 2011); however, its role in HS adaptation is not fully understood. Intriguingly, prolactin increases β cell proliferation and glucose-stimulated insulin secretion *in vivo* and *in vitro* (Brelje and Sorenson 1991; Hughes and Huang 2011). Thus, determining prolactin's role in HS metabolic adaptation is of obvious interest. An additional signal potentially involved in the insulin response during HS is LPS. Heat stress increases intestinal permeability to luminal content due to redistribution of blood flow from the viscera to the periphery in an attempt to maximize radiant heat dissipation (Lambert *et al.* 2002). The decrease in intestinal perfusion leads to mucosal hypoxia which compromises the intestinal barrier function (Hall *et al.* 1999). In agreement, we observe an increase in intestinal permeability and the subsequent increase in circulating LPS in our model (Pearce *et al.* 2013c; Sanz Fernandez *et al.* 2014b), and an LPS challenge acutely increases circulating insulin both *in vivo* (Burdick Sanchez *et al.* 2013) and *in vitro* (Vives-Pi *et al.* 2003). Interestingly, in the current study *CD14* gene expression in LD and circulating LBP, both key proteins in the recognition of LPS by Toll-like receptor 4 (Lu *et al.* 2008), were up-regulated and numerically increased, respectively, in HS pigs. Further, the partial loss of differences in basal and stimulated metabolism between treatments by d 6 of environmental exposure might correspond to the temporal pattern of acclimation by the intestinal barrier. This hypothesis is supported by the lack of differences in LBP by the end of the experiment. Moreover, because we utilized jugular catheters, we administered antibiotics throughout the length of the experiment to prevent infection. This experimental approach may have influenced the intestinal flora, mitigating the leakage of luminal pro-inflammatory molecules to the portal and systemic blood

stream, and/or the immune response to them. Determining how antibiotics influence the metabolic and inflammatory response to HS is of academic and practical interest.

Contrary to our previous studies in ruminants (O'Brien *et al.* 2010; Wheelock *et al.* 2010), the insulin response to the GTT was decreased by HS. This might be due to species differences in insulin responsiveness as pigs are generally considered more insulin sensitive than ruminants (Brockman and Laarveld 1986), and therefore, changes in peripheral insulin sensitivity might be adequate for insulin to exert its effects on HS adaptation in swine. The effects of HS on whole-body insulin sensitivity is not clear as there is evidence that it either increases, does not change or actually decreases (see review (Baumgard and Rhoads 2013). In the current study, the rate of glucose disposal following the GTT was decreased by HS and this suggests insulin resistance, but this is likely explained by a decreased insulin response to the GTT in HS pigs. Coupling the insulin response with the glucose disposal parameters (the insulinogenic index) suggests that HS pigs actually required less insulin to stimulate a similar quantity of systemic glucose uptake. The increased insulin sensitivity agrees with previous reports where thermal therapy improved insulin action in diabetic and obese rodents (Kokura *et al.* 2007; Gupte *et al.* 2009) and humans (Hooper 1999), and might be the result of heat shock protein up-regulation, as over-expression of HSP72 protects against obesity-induced insulin resistance and HSP co-inducers improve insulin sensitivity (Chung *et al.* 2008). Consequently, both the increase in basal circulating insulin and the apparent enhanced insulin sensitivity seem to be critical for survival and adaptation to a heat load as diabetics are more susceptible to heat related-illness/death and insulin administration to diabetic rodents improves survivability to severe HS (Semenza *et al.* 1999; Niu *et al.* 2003).

Heat stress appears to markedly alter intracellular energetics, characterized by a decrease in ATP production via oxidative phosphorylation and an increase in energy production via aerobic glycolysis (Baumgard and Rhoads 2013) and this resembles the Warburg effect utilized by cancer cells (Kim and Dang 2006). Our molecular data agrees with this tenet as the gene expression of the tricarboxylic acid cycle (*IDH2*) and electron transport chain enzymes (*NDUFB7, NDUFS7*), as well as mitochondrial creatine kinase (*CKMT2*; responsible for the transfer of high energy phosphate from the mitochondria to the cytosol) were down-regulated in LD and liver of HS compared to PFTN pigs. In contrast to our previous findings (Sanders *et al.* 2009; Won *et al.* 2012), LD pyruvate dehydrogenase kinase 4 (*PDK4*), which inactivates the pyruvate dehydrogenase complex and thus regulates glucose flux from glycolysis into the TCA cycle, was down-regulated in HS pigs. Reasons for these dissimilar results may include differences in experimental design, as in the past we have compared HS animals to TN *ad libitum* controls instead of to PFTN ones, and feed restriction by itself increases PDK4 expression (Furuyama *et al.* 2003). Nevertheless, the pyruvate dehydrogenase complex is regulated by several PDK isozymes as well as pyruvate dehydrogenase phosphatases (Harris *et al.* 2002), so further research is required in order to establish its role in the cellular metabolic shift during HS.

The decreased AT mobilization and improved insulin sensitivity observed in the HS pigs might actually be the result of reduced energetic requirements. Traditionally, HS has been thought to increase whole-body energy expenditure due to employing heat dissipation mechanisms and enhanced chemical reactions rate (e.g. Van't Hoff-Arrhenius equation; Fuquay 1981). Further, the potential activation of the immune system as a consequence of the HS-induced increased intestinal permeability comes with a high energetic cost. In an apparent

contrast, heat-stressed animals experience a marked decrease in thyroid hormones, which are typically correlated with whole-body energy expenditure and heat production (as reviewed in (Baumgard and Rhoads 2013). In our study, after only 24 h of HS exposure, both circulating T_3 and T_4 were sharply decreased, which suggests that in the early stages of HS the reduction in thyroid hormones is related to reduced thyroid gland activity. However, T_4 progressively increased to baseline levels while T_3 and similarly T_3:T_4 ratio remained low throughout the experiment. This implies that, as HS progresses, the thyroid gland acclimates, while the extrathyroidal T_4 to T_3 conversion (responsible for most of the circulating T_3 in mammals; Kahl *et al.* 2002) remains reduced. In agreement, by d 8 of HS, hepatic 5'D activity was decreased in HS pigs compared to PFTN animals. The mechanism by which HS decreases thyroid parameters are not fully understood, but both cortisol and LPS have been shown to regulate aspects of the thyroid axis in different models (Kahl *et al.* 2000; Larson 2005) and both are increased in acute HS (Baumgard and Rhoads 2013). Determining the energetic and nutrient demand of heat-stressed animals would presumably provide clues on how to better treat humans and animals suffering from heat-related illnesses.

In summary, HS represents a major environmental hazard compromising both human health and animal agriculture. In the current study, we demonstrate that heat-stressed pigs experience increased basal insulin secretion and whole body insulin sensitivity, and both variables likely prevent adipose tissue mobilization. The similarities with our observations during both growth and lactation in monogastrics and ruminants, coupled with the thermal therapy and diabetic models suggest that these responses are conserved amongst species, highlighting its importance in the adaptation to a thermal load. A better understanding of the

physiological effects of HS is essential in order to develop treatment protocols and mitigation strategies both for human health and animal agriculture.

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Disclosure Statement

The authors are not aware of any affiliation, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

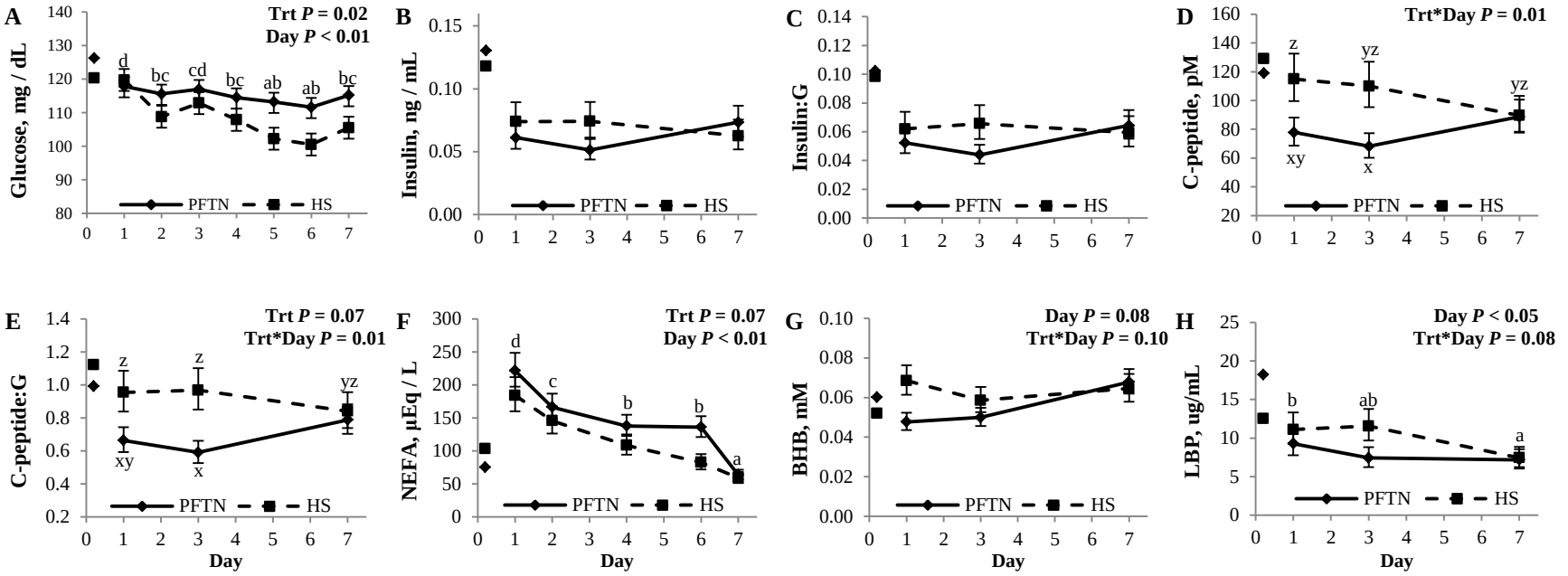

Figure 1. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on temporal changes in plasma (A) glucose, (B) insulin, (C) insulin to glucose ratio (Insulin:G) (D) C-peptide, (E) C-peptide to glucose ratio (C-peptide:G), (F) non-esterified fatty acids (NEFA), (G) β-hydroxybutyrate (BHB), and (H) lipopolysaccharide binding protein (LBP). Values on day 0 represent period 1 average that was statistically used as covariate. [a-d]Means with different superscript differ ($P \leq 0.05$). [x-z]Days with different superscript differ ($P \leq 0.05$).

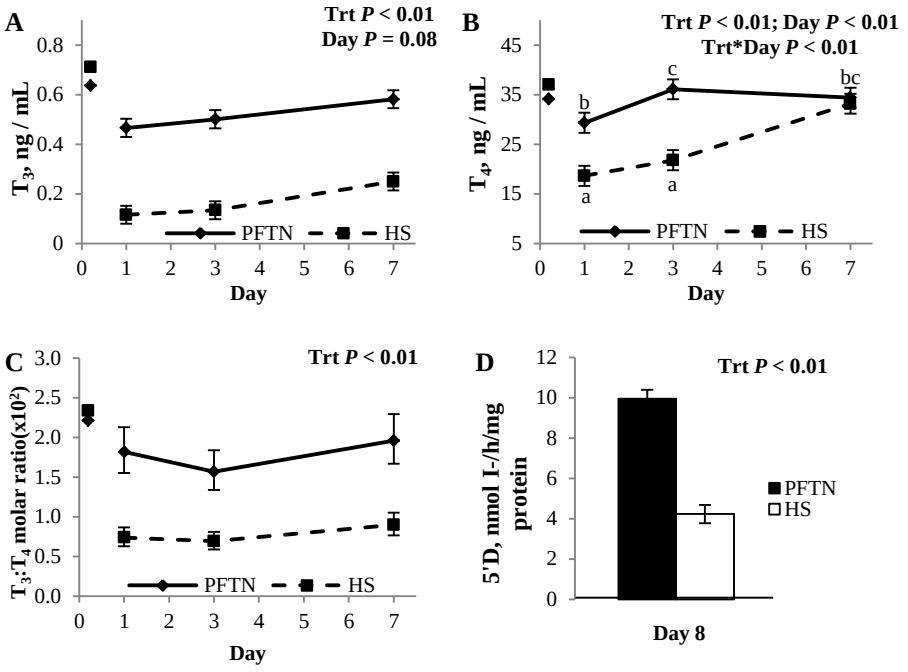

Figure 2. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on temporal changes in plasma (A) T_3, (B) T_4, (C) T_3 to T_4 ratio (T_3:T_4); and (D) hepatic 5'-deiodinase type I activity (5'D) after 8 days of environmental treatment. Values on day 0 represent period 1 average that was statistically used as covariate. $^{a\text{-}c}$Means with different superscript differ ($P \leq 0.05$).

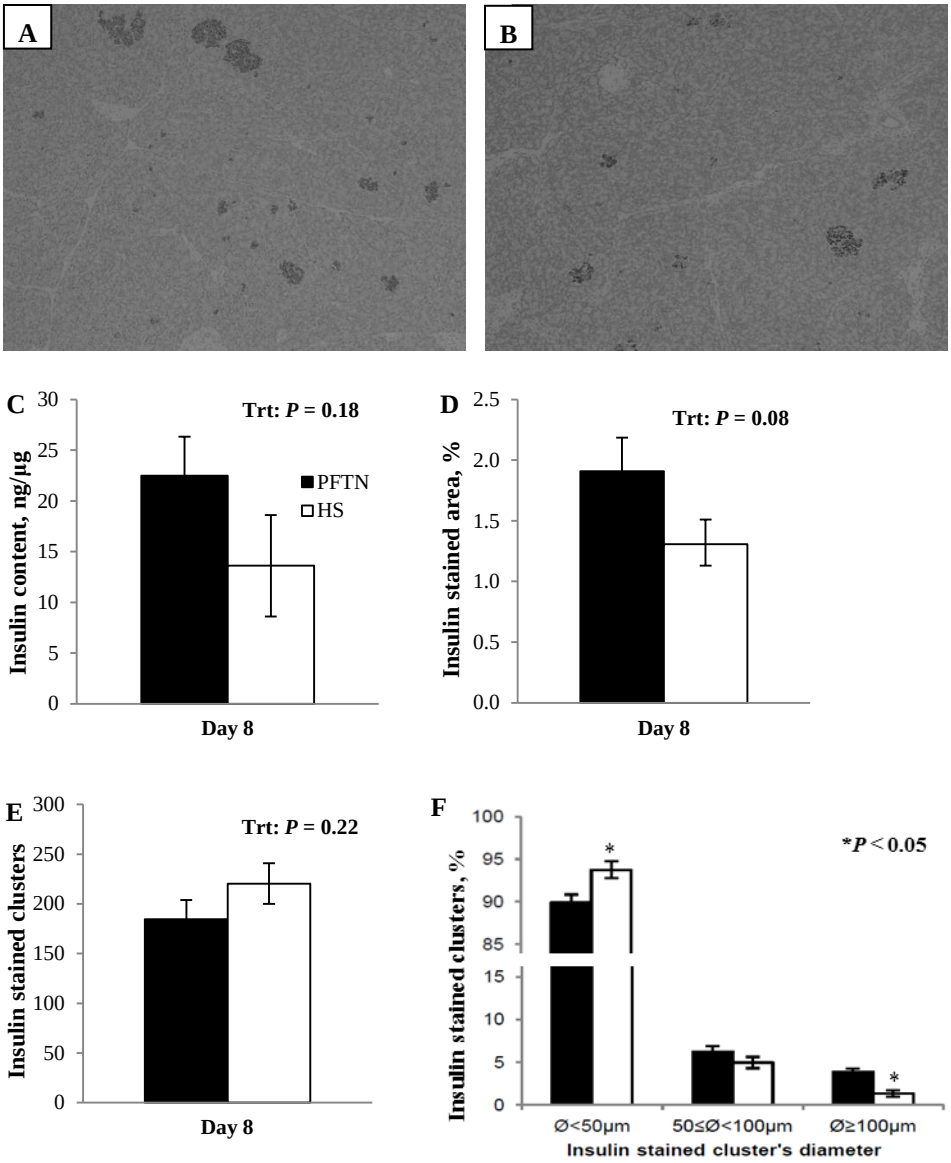

Figure 3. Effects of 8 days of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on pancreatic insulin content. (A) Representative picture of PFTN pancreas; (B) representative picture of PFTN pancreas; (C) insulin content; (D) insulin stained area; (E) number of insulin stained clusters; (F) insulin stained clusters size distribution. *Represents differences with PFTN controls ($P \leq 0.05$).

Table 3. Effects of pair-feeding or heat stress on body temperature indices and feed intake

	Day										*P*		
	P1*	1	2	3	4	5	6	7	8	SEM	Trt[1]	Day	TXD[2]
Tr[3], °C:													
PFTN[4]	39.32	39.08^{c}	38.95^{b}	38.92^{b}	38.74^{a}	38.82^{ab}	38.83^{ab}	38.82^{ab}	38.82^{ab}	0.10	<0.01	<0.01	<0.01
HS[5]	39.36	40.06^{d}	40.73^{g}	40.50^{f}	40.31^{e}	40.26^{de}	40.29^{de}	40.27^{de}	40.26^{de}	0.12			
RR, bpm[6]:													
PFTN	40	34^{b}	23^{a}	21^{a}	21^{a}	19^{a}	22^{a}	24^{a}	22^{a}	4	<0.01	<0.01	<0.01
HS	32	88^{c}	118^{f}	98de	100de	96^{cd}	105^{e}	98de	92de	5			
FI[7], kg:													
PFTN	2.01	1.20^{x}	1.16^{x}	1.20^{x}	1.15^{x}	1.19^{x}	1.18^{x}	1.55^{y}	1.23^{x}	0.05	0.75	<0.01	0.99
HS	1.97	1.19^{x}	1.14^{x}	1.19^{x}	1.14^{x}	1.18^{x}	1.16^{x}	1.53^{y}	1.21^{x}	0.06			

[1]Treatment
[2]Treatment by day interaction
[3]Rectal temperature
[4]Pair-fed thermoneutral
[5]Heat stress
[6]Respiration rate, breaths per minute
[7]Feed intake
*Represents period 1 values that were statistically used as covariate
[a-f]Means with different superscript differ ($P \leq 0.05$)
[x,y]Days with different superscript differ ($P \leq 0.05$)

Table 4. Effects of pair-feeding or heat stress on the response to metabolic challenges

	P[1]1*		Day 1-2 of P2		Day 6-7 of P2			*P*		
Parameter	PFTN[2]	HS[3]	PFTN	HS	PFTN	HS	SEM	Trt[4]	Day	TXD[5]
Glucose tolerance test:										
Glucose AUC[6], mg/dL·min	1991	1712	2257^{a}	2595^{b}	2273^{a}	2189^{a}	97	0.32	0.01	0.01
Glucose delta, mg/dL	172.6	171.4	186.1^{ab}	198.3^{b}	190.6^{ab}	178.6^{a}	7.5	0.99	0.18	0.04
Glucose disappearance, mg/dL/min	8.50	8.98	7.54	6.69	8.61	7.44	0.48	0.07	0.06	0.72
Insulin AUC, ng/mL·min	9.06	7.52	8.82	6.16	6.75	6.13	0.53	0.02	0.06	0.06
Insulin delta, ng/mL	0.58	0.54	0.47	0.39	0.43	0.36	0.03	0.06	0.11	0.89
Insulinogenic index, AU	5.20	4.85	4.53^{b}	2.50^{a}	3.68^{a}	3.03^{a}	0.43	0.04	0.47	0.01
Epinephrine challenge:										
NEFA AUC, µEq/L·min	812	1260	1717	1309	1332	381	198	0.01	<0.01	0.17
Glucose AUC, mg/dL·min	519	433	620	580	689	685	77	0.80	0.23	0.80

[1]Period
[2]Pair-fed thermoneutral
[3]Heat stress
[4]Treatment
[5]Treatment by day interaction
[6]Area under the curve
*Represents period 1 values that were statistically used as covariate
[a,b]Means with different superscript differ ($P \leq 0.05$)

Table 5. Effects of 8 days of pair-feeding or heat stress on gene expression in adipose tissue

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt[1]				*P*
			PFTN[2]	HS[3]	SEM	Fdiff[4]	Trt[5]
ADRBK1	Adrenergic, beta, receptor kinase 1	F[6]:GCGTCATGCAGAAGTACCTG R[7]:GCTTCAGGCAGAAGTCTCGG	-1.17	-0.53	0.28	-1.56	0.13
ATGL (PNPLA2)	Adipose triglyceride lipase (Patatin-like phospholipase domain containing 2)	F:GTGGCCACGGCCCTGGTTAC R:CGGTTCTTGGGCCCACTGCA	-0.43	0.21	0.23	-1.56	0.07
ATP5J2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2	F:AGATGACGTCAGTTGTACCGC R:ATGCCCGAAGGGGTGAAATC	0.69	0.28	0.30	1.33	0.35
CAPN1	Calpain 1, (mu/I) large subunit	F:GAGCTGTTCTCAAACCCCCA R:GGGTGTCGTTGAGGGTAAGG	-0.95	-0.50	0.22	-1.37	0.16
CD14	CD14 molecule	F:CCTTGCAAGATCCTTCCGGT R:TTGGCAGACTTTGGGGGTTT	0.10	-0.73	0.46	1.78	0.23
CIRBP	Cold inducible RNA binding protein	F:GAGTCAGGGTGGCAGCTATG R:ACCCTTCTGAGTTGCACTGG	0.70	0.12	0.31	1.49	0.21
FADS1	Fatty acid desaturase 1	F:CGTGATTGACCGGAAGGTGT R:ACAAGGCCCTGGTTGATGTG	-2.26	-2.16	0.34	-1.07	0.83
FASN	Fatty acid synthase	F:CTCATCGGCGGTGTGGACAT R:CATCGTGTTCGCCTGCTTGG	0.69	0.90	0.37	-1.16	0.69
G6PD	Glucose-6-phosphate dehydrogenase	F:GCGATGCTTTCCATCAGTCG R:GCGTAGCCCACGATGTATGT	1.06	1.62	0.36	-1.47	0.29
GSK3B	Glycogen synthase kinase 3 beta	F:CGAGACACACCTGCACTCTT R:TGACGCAGAAGCGGTGTTAT	-0.62	-0.18	0.29	-1.36	0.30
HSF1	Heat shock transcription factor 1	F:TTCAAGCACAGCAACATGGC R:CACGCTGGTCACTTTCCTCT	-0.51	-0.19	0.26	-1.25	0.40
HSF2	Heat shock transcription factor 2	F:AGGCCAGGATGACTTGTTGG R:ACACCTCCTTCCAAAGGGAC	-0.64	-1.12	0.41	1.39	0.43
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	F:ATCGCCCAGTTGATGTCGTT R:TATCGTGAGGGTCCGGTCTT	0.21	-0.73	0.41	1.92	0.13
HSPA2	Heat shock 70kDa protein 2	F:TGAGAGTTTCCAGAAGGCGG R:AAGACGAGCAAGCGACGTTA	0.02	-0.92	0.55	1.92	0.25
HSPA4	Heat shock 70kDa protein 4	F:CGCTTCGCAGTGTTTTGGAA R:TGCACAGCCTCGAGTAACAG	0.05	-1.23	0.48	2.43	0.08
HSPB8	Heat shock 22kDa protein 8	F:GATGGCTACGTGGAGGTGTC R:GGGGAAAGCGAGGCAAATAC	-0.09	-1.42	0.53	2.51	0.10
HSPCB	Heat shock protein 90kDa alpha (cytosolic), class B member 1	F:CCGTTCTCTTGAGTCACCCC R:GAGACATGAGCTGGGCGATT	0.19	0.01	0.27	1.13	0.66
HSPE1	Heat shock 10kDa protein 1 (chaperonin 10)	F:AGCTGTTGGATCAGGCTCTAAA R:TGTGATGCCATTAGACAGTGAC	1.76	0.40	0.52	2.57	0.09
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	F:CGACCAGAGGATCAAGGTGG R:GGGAGCCCCAGGTCAAAATA	0.06	0.18	0.16	-1.09	0.59
INSR	Insulin receptor	F:CAGCCTGCGAGAGCGGATCG R:TGAGAATAACCCCGGCCGCC	-0.97	-0.42	0.19	-1.46	0.07
LDHA	Lactate dehydrogenase A	F:AAGGAACACTGGAAAGCGG R:CATGGTGGAAATCGGATGCAC	1.10	0.56	0.47	1.45	0.44
MAPK14	Mitogen-activated protein kinase 14	F:CAGGGGCTGAGCTTTTGAAG R:GCAAGTCAACAGCCAAGGGA	-0.07	-0.39	0.24	1.25	0.35
MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	F:TTCTTGCTGCCAGCTCGTTT R:AGCACAGCTACCTTGGCATT	-0.05	0.46	0.41	-1.42	0.39
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	F:ATCAGCCCGGACAGGATCTA R:GCCGAGAGCAAAGGAGTCTT	0.02	0.70	0.27	-1.60	0.10
MKNK2	MAP kinase interacting serine/threonine kinase 2	F:AAGAAGAAGAGGTGCCGAGC R:CACCTCCCGGAAAACCCTAC	-0.65	-0.10	0.25	-1.46	0.15
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	F:CGCATGAAGGAGTTTGAGCG R:GGGGCCTGGAGGCTTTTATT	-0.03	0.53	0.23	-1.47	0.12
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa	F:TGCCAGAGCCTCGTTATGTT R:CAGTCTCTTCTCCCGCTTGA	0.13	0.42	0.23	-1.22	0.40
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	F:CGCGAGTATCTGTGCGTTTC R:TTCAGTCTCCAGTCATGGCG	-0.17	0.32	0.22	-1.40	0.13
PC	Pyruvate carboxylase	F:AAGCCCTGGCCATAAGTGAC R:CCCAATCTGGCCCTTCACAT	0.30	0.48	0.24	-1.13	0.60
PDAP1	PDGFA associated protein 1	F:GTGGAAGGGCTCATCGACAT R:GCTCTGTCTTCCCCGCTAAA	0.61	1.40	0.28	-1.73	0.07
PDCD7	Programmed cell death 7	F:TCTTACAGCCTTTCCGGCAG R:TTAATGGCGGTTGCCCAGAT	-1.17	-1.22	0.37	1.04	0.92
PEMT	Phosphatidylethanolamine N-methyltransferase	F:ATGGAGCGCGTGTTTGACTA R:TCCTGGGATCTCGTTCTCGT	0.32	0.40	0.27	-1.06	0.84
PLIN	Perilipin	F:CAACAAGGGCCTGACTTTGC R:ATTGCATACAGACGCCACCA	-1.63	-0.44	0.71	-2.28	0.26

Table 5. (Continued)

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt		SEM	Fdiff	*P*
			PFTN	HS			Trt
PRKAG1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	F:GCATCCTCAAGACACCCCAG R:GCAGCTCGGACACCATTAGT	-0.59	0.33	0.30	-1.89	0.05
PRLR	Prolactin receptor	F:ACAGTCACCTCCGGGAAAAC R:TAGGGCCGCCAGTTTTGTAG	-1.15	-0.92	0.63	-1.17	0.79
RBM7	RNA binding motif protein 7	F:AGCAGGTACGAAAGAACGGT R:GGGACTGAACTGATGGCGAA	-0.13	-0.72	0.31	1.51	0.21
UBE2B	Ubiquitin-conjugating enzyme E2B	F:GGTGACCCACAGTGATTCGG R:AGGTGGGTCCTCTTGCAATC	-1.03	-1.48	1.42	1.37	0.81
UBE2G1	Ubiquitin-conjugating enzyme E2G 1	F:TCGCCCGCTGTGTAAGAAAA R:CAGTTCCAGCCAGTGTTTGC	0.92	0.19	0.70	1.66	0.47
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	F:CAACTTTGGCTCTGTTCGCC R:CACGTTGCTGACAGGGATCT	0.35	1.54	0.57	-2.28	0.19
ZSCAN29	Zinc finger and SCAN domain containing 29	F:CCCACAGGAGAAGCTCAGAC R:CCCAGTGATCCTGTTCCACC	-1.13	-0.31	0.26	-1.77	0.05

[1]Delta delta Ct
[2]Pair-fed thermoneutral
[3]Heat stress
[4]Fold difference: positive/negative values indicate increased/decreased transcript abundance in HS pigs compared to PFTN controls
[5]Treatment
[6]Forward
[7]Reverse

Table 6. Effects of 8 days of pair-feeding or heat stress on gene expression in skeletal muscle (longissimus dorsi)

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt[1]				*P*
			PFTN[2]	HS[3]	SEM	Fdiff[4]	Trt[5]
ADRBK1	Adrenergic, beta, receptor kinase 1	F[6]:GCGTCATGCAGAAGTACCTG R[7]:GCTTCAGGCAGAAGTCTCGG	1.28	1.37	0.24	-1.06	0.80
ATP5J2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2	F:GATGACGTCAGTTGTACCGC R:AATGCCCGAAGGGGTGAAAT	0.25	0.51	0.35	-1.20	0.60
CAPN1	Calpain 1, (mu/I) large subunit	F:TGCACCGAGTAGTTCCACAC R:AACTCATTGCCTTGGGCAGA	0.67	0.70	0.38	-1.02	0.96
CD14	CD14 molecule	F:AACCCCCAAAGTCTGCCAAA R:AAGGTCCTCAAAGCCTCTGC	0.83	0.08	0.17	1.68	0.01
CIRBP	Cold inducible RNA binding protein	F:GAGTCAGGGTGGCAGCTATG R:ACCCTTCTGAGTTGCACTGG	0.27	0.43	0.30	-1.12	0.71
CKMT2	Creatine kinase, mitochondrial 2 (sarcomeric)	F:CTAACTGGCCGCAATGCTTC R:GCAGTTGTTGTGCTTGCGTA	0.87	1.89	0.41	-2.03	0.10
CPT2	Carnitine palmitoyltransferase 2	F:TCAGGCGGTAAGATGTGGAA R:ACCATTACCTGAAGAGCCCG	-0.35	-0.03	0.29	-1.25	0.45
DDB1	Damage-specific DNA binding protein 1, 127kDa	F:AAGCGCCTACCATCTGCTTT R:TCTGGCACTGCAATCACCAT	1.10	1.32	0.23	-1.16	0.51
FAR1	Fatty acyl CoA reductase 1	F:TCCAACAATGCCCTTGCAGA R:GGGCCTTATGGCTTACAGCA	0.25	0.18	0.25	1.05	0.83
G6PD	Glucose-6-phosphate dehydrogenase	F:GCGATGCTTTCCATCAGTCG R:GCGTAGCCCACGATGTATGT	1.04	1.25	0.16	-1.16	0.35
HIF1a	Hypoxia inducible factor 1, alpha subunit	F:ATCTCGGGCACAGATTCGC R:TCCTCACACGCAAATAGCTGA	0.10	-0.19	0.21	1.22	0.34
HSF1	Heat shock transcription factor 1	F:TTCAAGCACAGCAACATGGC R:CACGCTGGTCACTTTCCTCT	1.99	1.94	0.30	1.04	0.90
HSF2	Heat shock transcription factor 2	F:AGGCCAGGATGACTTGTTGG R:ACACCTCCTTCCAAAGGGAC	0.46	0.35	0.24	1.08	0.76
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	F:ATCGCCCAGTTGATGTCGTT R:TATCGTGAGGGTCCGGTCTT	1.02	-0.05	0.18	2.10	<0.01
HSPA2	Heat shock 70kDa protein 2	F:TGAGAGTTTCCAGAAGGCGG R:AAGACGAGCAAGCGACGTTA	-0.06	-0.01	0.53	-1.04	0.94
HSPA4	Heat shock 70kDa protein 4	F:AAAGATTCCATGGCCGAGCA R:GCCGTCTCCTTCAGTTTGGA	0.88	0.28	0.19	1.52	0.05
HSPB8	Heat shock 22kDa protein 8	F:GATGGCTACGTGGAGGTGTC R:GGGGAAAGCGAGGCAAATAC	1.33	0.91	0.22	1.34	0.20
HSPCB	Heat shock protein 90kDa alpha (cytosolic), class B member 1	F:AGCCGTTCTCTTGAGTCACC R:TGCCTGGAAGGCAAAAGTCT	0.79	0.01	0.20	1.72	0.02
HSPE1	Heat shock 10kDa protein 1 (chaperonin 10)	F:TTGCAAGCAACCGTAGTAGC R:ACAGTGACTTGTTTCAGTCTACG	0.17	-1.08	0.34	2.38	0.02
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	F:CGACCAGAGGATCAAGGTGG R:GGGAGCCCCAGGTCAAAATA	0.73	1.55	0.28	-1.77	0.06
KCTD6	Potassium channel tetramerization domain containing 6	F:TGGATAATGGAGACTGGGGCT R:AGCATGGAATCCGGGTAACG	0.75	0.90	0.34	-1.11	0.76
LDHA	Lactate dehydrogenase A	F:TTGTTGGGGTTGGTGCTGTT R:TGGGGTCCTAAGGAAAAGGC	1.14	1.83	0.43	-1.61	0.28
MAPK14	Mitogen-activated protein kinase 14	F:AAGACTCGTTGGAACCCCAG R:TCCAGCAAGTCAACAGCCAA	1.41	1.14	0.24	1.21	0.43
MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	F:TTTCTTGCTGCCAGCTCGTT R:GCACAGCTACCTTGGCATTG	1.46	2.37	0.41	-1.88	0.14
MEF2A	Myocyte enhancer factor 2A	F:AAGGAACACAGAGGGTGCG R:AAAGCATTAGGGCTGGTCACA	0.64	0.72	0.24	-1.06	0.81
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	F:ATCAGCCCGGACAGGATCTA R:GCCGAGAGCAAAGGAGTCTT	-0.07	-0.23	0.19	1.12	0.58
MKNK2	MAP kinase interacting serine/threonine kinase 2	F:AAGAAGAAGAGGTGCCGAGC R:CACCTCCCGGAAAACCCTAC	1.13	1.40	0.28	-1.21	0.51
MYOD1	Myogenic differentiation 1	F:CTACAGCGGTGACTCAGACG R:AATAGGTGCCGTCGTAGCAG	1.24	1.52	0.33	-1.21	0.57
NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	F:CAGAGCCTTGCATGTCGGTA R:AGCTAACCAATCCTGGTGGC	0.59	1.08	0.32	-1.40	0.29
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	F:CGCATGAAGGAGTTTGAGCG R:GGGGCCTGGAGGCTTTTATT	0.10	0.47	0.34	-1.29	0.45
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa	F:TGCCAGAGCCTCGTTATGTT R:CAGTCTCTTCTCCCGCTTGA	0.28	1.29	0.36	-2.01	0.06
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	F:CGCGAGTATCTGTGCGTTTC R:TTCAGTCTCCAGTCATGGCG	1.21	1.88	0.32	-1.59	0.16
PC	Pyruvate carboxylase	F:AAGCCCTGGCCATAAGTGAC R:CCCAATCTGGCCCTTCACAT	2.65	3.08	0.35	-1.35	0.39

Table 6. (Continued)

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt PFTN	Trt, ΔΔCt HS	SEM	Fdiff	*P* Trt
PDAP1	PDGFA associated protein 1	F:GTGGAAGGGCTCATCGACAT R:GCTCTGTCTTCCCCGCTAAA	0.56	0.51	0.28	1.04	0.91
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	F:AAGCCACATTGGCAGCATTG R:GGTGTTCGACTGTAGCCCTC	-0.78	0.56	0.44	-2.53	0.05
PEMT	Phosphatidylethanolamine N-methyltransferase	F:ATGGAGCGCGTGTTTGACTA R:TCCTGGGATCTCGTTCTCGT	2.55	3.11	0.33	-1.47	0.26
PRKAG1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	F:GCATCCTCAAGACACCCCAG R:GCAGCTCGGACACCATTAGT	0.59	0.85	0.25	-1.20	0.48
RASA1	RAS p21 protein activator (GTPase activating protein) 1	F:GGGAAGGGCAAAACCCTGTA R:GCTCAATTGGCAGCGCATAA	0.11	0.05	0.16	1.04	0.81
RBM7	RNA binding motif protein 7	F:GCAGGTACGAAAGAACGGTG R:ATGGGACTGAACTGATGGCG	0.27	0.16	0.24	1.08	0.76
SLC16A5	Solute carrier family 16, member 5	F:TCGGCATCTTCTTCACCGAA R:GATGAATCCTGCCGTGAGGT	1.23	2.61	0.40	-2.60	0.03
SLC25A27	Solute carrier family 25, member 27	F:AAGCTCTGGCAAGGAGTGAC R:CACGAAATCGCAACGGCTTT	0.76	0.07	0.29	1.61	0.12
UBE2G1	Ubiquitin-conjugating enzyme E2G 1	F:TCGCCCGCTGTGTAAGAAAA R:CAGTTCCAGCCAGTGTTTGC	0.26	0.36	0.35	-1.07	0.83
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	F:CAACTTTGGCTCTGTTCGCC R:CAGTTCCAGCCAGTGTTTGC	-0.05	0.40	0.33	-1.37	0.35
ZSCAN29	Zinc finger and SCAN domain containing 29	F:CCCACAGGAGAAGCTCAGAC R:CCCAGTGATCCTGTTCCACC	1.26	1.06	0.16	1.15	0.39

[1]Delta delta Ct
[2]Pair-fed thermoneutral
[3]Heat stress
[4]Fold difference: positive/negative values indicate increased/decreased transcript abundance in HS pigs compared to PFTN controls
[5]Treatment
[6]Forward
[7]Reverse

Table 7. Effects of 8 days of pair-feeding or heat stress on gene expression in liver

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt[1]		SEM	Fdiff[4]	*P*
			PFTN[2]	HS[3]			Trt[5]
ADRBK1	Adrenergic, beta, receptor kinase 1	F[6]:GCGTCATGCAGAAGTACCTG R[7]:GCTTCAGGCAGAAGTCTCGG	0.18	0.02	0.18	1.12	0.54
ATP5J2	ATP synthase, H+ transporting, mitochondrial Fo complex, subu F2	F:GATGACGTCAGTTGTACCGC R:AATGCCCGAAGGGGTGAAAT	0.08	0.34	0.12	-1.20	0.14
CAPN1	Calpain 1, (mu/I) large subunit	F:TGCACCGAGTAGTTCCACAC R:AACTCATTGCCTTGGGCAGA	0.52	0.70	0.59	-1.13	0.17
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	F:CCCGCTTCCAGAGGGATTTT R:GGGCTGGTAGGCTAGGATCT	0.94	1.49	0.34	-1.46	0.27
CD14	CD14 molecule	F:AACCCCCAAAGTCTGCCAAA R:AAGGTCCTCAAAGCCTCTGC	-0.32	0.08	0.26	-1.32	0.28
CIRBP	Cold inducible RNA binding protein	F:GAGTCAGGGTGGCAGCTATG R:ACCCTTCTGAGTTGCACTGG	0.13	0.18	0.13	-1.04	0.81
FADS2	Fatty acid desaturase 2	F:GCGCAGATGCCTACCTTT R:CTAAATCAAGGTCGCGGTGG	0.48	0.65	0.25	-1.13	0.64
FASN	Fatty acid synthase	F:TCATCGGCGGTGTGGACAT R:CCATCGTGTTCGCCTGCTTG	2.56	1.17	0.24	2.62	<0.01
G6PD	Glucose-6-phosphate dehydrogenase	F:GCGATGCTTTCCATCAGTCG R:GCGTAGCCCACGATGTATGT	1.56	0.99	0.46	1.48	0.39
GCK	Glucokinase (hexokinase 4)	F:CTGCCTTGGAAGCCTGTTGG R:ATCTCCTTCTGCATCCGCCT	2.36	1.82	0.64	1.45	0.56
HIF1a	Hypoxia inducible factor 1, alpha subunit	F:ATCTCGGGCACAGATTCGC R:TCCTCACACGCAAATAGCTGA	1.02	0.42	0.46	1.52	0.37
HSF1	Heat shock transcription factor 1	F:TTCAAGCACAGCAACATGGC R:CACGCTGGTCACTTTCCTCT	0.14	0.14	0.20	1.00	1.00
HSF2	Heat shock transcription factor 2	F:AGGCCAGGATGACTTGTTGG R:ACACCTCCTTCCAAAGGGAC	0.14	-0.07	0.17	1.16	0.40
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	F:ATCGCCCAGTTGATGTCGTT R:TATCGTGAGGGTCCGGTCTT	0.53	0.38	0.13	1.11	0.45
HSPA2	Heat shock 70kDa protein 2	F:TGAGAGTTTCCAGAAGGCGG R:AAGACGAGCAAGCGACGTTA	-0.46	-0.62	0.52	1.12	0.83
HSPA4	Heat shock 70kDa protein 4	F:AAAGATTCCATGGCCGAGCA R:GCCGTCTCCTTCAGTTTGGA	0.59	0.26	0.14	1.26	0.12
HSPB8	Heat shock 22kDa protein 8	F:GATGGCTACGTGGAGGTGTC R:GGGGAAAGCGAGGCAAATAC	0.28	0.39	0.18	-1.08	0.69
HSPCB	Heat shock protein 90kDa alpha (cytosolic), class B member 1	F:AGCCGTTCTCTTGAGTCACC R:TGCCTGGAAGGCAAAAGTCT	-0.10	-0.18	0.24	1.06	0.81
HSPE1	Heat shock 10kDa protein 1 (chaperonin 10)	F:TTGCAAGCAACCGTAGTAGC R:ACAGTGACTTGTTTCAGTCTACG	1.11	0.41	0.26	1.62	0.08
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	F:CGACCAGAGGATCAAGGTGG R:GGGAGCCCCAGGTCAAAATA	0.69	0.40	0.30	1.22	0.50
KCTD6	Potassium channel tetramerization domain containing 6	F:TGGATAATGGAGACTGGGGCT R:AGCATGGAATCCGGGTAACG	0.53	0.09	0.16	1.36	0.08
LDHA	Lactate dehydrogenase A	F:TTGTTGGGGTTGGTGCTGTT R:TGGGGTCCTAAGGAAAAGGC	0.74	0.76	0.20	-1.01	0.96
LIPG	Lipase, endothelial	F:CTGGTTCTGGTTCAAGCCCT R:GATCAGACAGTGGTGGCCTT	0.09	0.32	0.31	-1.17	0.60
MAPK14	Mitogen-activated protein kinase 14	F:CAGGGGCTGAGCTTTTGAAG R:GCAAGTCAACAGCCAAGGGA	0.84	0.34	0.29	1.41	0.25
MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	F:TTTCTTGCTGCCAGCTCGTT R:GCACAGCTACCTTGGCATTG	0.54	0.71	0.44	-1.13	0.80
MIF	Macrophage migration inhibitory factor	F:ATCAGCCCGGACAGGATCTA R:GCCGAGAGCAAAGGAGTCTT	0.67	0.59	0.31	1.06	0.85
MKNK2	MAP kinase interacting serine/threonine kinase 2	F:AAGAAGAAGAGGTGCCGAGC R:CACCTCCCGGAAAACCCTAC	0.73	0.38	0.23	1.27	0.29
NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	F:CAGAGCCTTGCATGTCGGTA R:AGCTAACCAATCCTGGTGGC	-0.91	-1.02	0.15	1.08	0.60
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	F:CGCATGAAGGAGTTTGAGCG R:GGGGCCTGGAGGCTTTTATT	0.12	0.48	0.12	-1.28	0.05
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa	F:TGCCAGAGCCTCGTTATGTT R:CAGTCTCTTCTCCCGCTTGA	0.02	0.39	0.16	-1.29	0.10
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	F:CGCGAGTATCTGTGCGTTTC R:TTCAGTCTCCAGTCATGGCG	0.10	0.41	0.22	-1.24	0.32
PC	Pyruvate carboxylase	F:AAGCCCTGGCCATAAGTGAC R:CCCAATCTGGCCCTTCACAT	-0.38	-0.34	0.20	-1.03	0.87
PDAP1	PDGFA associated protein 1	F:GTGGAAGGGCTCATCGACAT R:GCTCTGTCTTCCCCGCTAAA	1.10	0.90	0.32	1.15	0.66

Table 7. (Continued)

Gene	Description	Primers, 5'-3'	Trt, ΔΔCt PFTN	Trt, ΔΔCt HS	SEM	Fdiff	*P* Trt
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	F:AAGCCACATTGGCAGCATTG R:GGTGTTCGACTGTAGCCCTC	-0.28	-0.46	0.14	1.13	0.37
PEMT	Phosphatidylethanolamine N-methyltransferase	F:ATGGAGCGCGTGTTTGACTA R:TCCTGGGATCTCGTTCTCGT	-0.57	-0.43	0.17	-1.10	0.56
POLD4	Polymerase (DNA-directed), delta 4, accessory subunit	F:GCTCTGCTGTGAAGTTTGGC R:AGCCTTTGGAAGGGTCATGG	0.13	0.14	0.13	1.00	0.90
PRKAG1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	F:GCATCCTCAAGACACCCCAG R:GCAGCTCGGACACCATTAGT	0.38	0.05	0.28	1.26	0.43
RBM7	RNA binding motif protein 7	F:AGCAGGTACGAAAGAACGGT R:GGGACTGAACTGATGGCGAA	0.33	-0.06	0.13	1.31	0.06
SDHC	Succinate dehydrogenase complex, subunit. C, 15kDa	F:CCGTGCCCATCTTAGTCCTC R:GGGGAGACAAAGGACGGTTT	0.59	0.55	0.21	1.03	0.89
SLC16A5	Solute carrier family 16, member 5	F:TCGGCATCTTCTTCACCGAA R:GATGAATCCTGCCGTGAGGT	0.15	0.14	0.34	1.01	0.98
SLC25A27	Solute carrier family 25, member 27	F:AAGCTCTGGCAAGGAGTGAC R:CACGAAATCGCAACGGCTTT	-0.02	-0.23	0.19	1.16	0.44
UBE2G1	Ubiquitin-conjugating enzyme E2G 1	F:ACTCGCCTGCTAATGTGGAC R:GTGCAGGAAAAACAGTGCCA	0.45	0.16	0.34	1.22	0.56
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	F:CAACTTTGGCTCTGTTCGCC R:CACGTTGCTGACAGGGATCT	-0.07	0.04	0.16	-1.08	0.63
ZSCAN29	Zinc finger and SCAN domain containing 29	F:CCCACAGGAGAAGCTCAGAC R:CCCAGTGATCCTGTTCCACC	-0.24	-0.43	0.21	1.14	0.54

[1]Delta delta Ct
[2]Pair-fed thermoneutral
[3]Heat stress
[4]Fold difference: positive/negative values indicate increased/decreased transcript abundance in HS pigs compared to PFTN controls
[5]Treatment
[6]Forward
[7]Reverse

CHAPTER 3: EFFECTS OF HEAT STRESS ON INSULIN SENSITIVITY

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Authors contributions:

- M.V.S.F. and L.H.B. designed the experiment, interpreted the data, drafted the manuscript, and prepared the figures.
- M.V.S.F., J.S.J., M.A., S.K.S., J.T.S., E.A.N., and L.H.B. conducted the live phase of the experiment.
- M.V.S.F. and E.A.N. performed the laboratory work.
- R.P.R. provided intellectual contributions key to the success of the experiment.
- All authors contributed to revision of the manuscript and approved this version.

Abstract

Proper insulin homeostasis appears critical for adapting to and surviving a heat load. Further, heat stress (HS) induces phenotypic changes in livestock that suggest an increase in insulin action. The current study objective was to evaluate the effects of HS on whole-body insulin sensitivity. Female pigs (57±4 kg body weight) were subjected to two experimental periods. During period 1, all pigs remained in thermoneutral conditions (TN; 21°C) and were

fed *ad libitum*. During period 2, pigs were exposed to: 1) constant HS conditions (32°C) and fed *ad libitum* (n=6), or 2) TN conditions and pair-fed (PFTN; n=6) to eliminate the confounding effects of dissimilar feed intake. A hyperinsulinemic euglycemic clamp (HEC) was conducted on d3 of both periods; and skeletal muscle and adipose tissue biopsies were collected prior to and after an insulin tolerance test (ITT) on d5 of period 2. During the HEC, insulin infusion increased circulating insulin and decreased plasma C-peptide and non-esterified fatty acids, similarly between treatments. From period 1 to 2, the rate of glucose infusion in response to the HEC remained similar in HS pigs while it decreased (36%) in PFTN controls. Prior to the ITT, HS increased (41%) skeletal muscle insulin receptor substrate-1 protein abundance, but did not affect protein kinase B or their phosphorylated forms. In adipose tissue, HS did not alter any of the basal or stimulated measured insulin signaling markers. In summary, HS increases whole-body insulin-stimulated glucose uptake, which may be partially explained by an increase in skeletal muscle insulin signaling.

Keywords: Heat Stress, Pig, Insulin Sensitivity, Metabolism

Introduction

Heat stress (HS) is a major environmental hazard for both humans and animals. Despite advances in the understanding of heat-related illnesses, there is no treatment against specific aspects of their pathophysiology, and protocols are limited to generic cooling and rehydration (Leon and Helwig 2010a). Therefore, a better understanding of the biological consequences of HS is critical in order to develop effective treatment protocols and mitigation strategies.

Interestingly, diabetic humans and rodents are more susceptible to heat-related illnesses, and exogenous insulin rescues this phenotype (Semenza *et al.* 1999; Niu *et al.* 2003). Further, thermal therapy improves insulin sensitivity in diabetic and obese rodents and humans (Hooper 1999; Kokura *et al.* 2007; Gupte *et al.* 2009). Moreover, we have previously reported that, despite hypercatabolic hallmarks including marked hypophagia and weight loss, HS increases basal and stimulated circulating insulin and decreases adipose tissue mobilization in a variety of species (Baumgard and Rhoads 2013), including pigs (Pearce *et al.* 2013a; Sanz Fernandez *et al.* 2014a).

An increase in insulin action might explain the increase in whole-body glucose utilization typically observed during HS (Febbraio 2001). Based on sheer mass, skeletal muscle is likely the main glucose sink during hyperthermia. However, the immune system might also consume a considerable amount of glucose (Greiner *et al.* 1994; Maciver *et al.* 2008), as we and others have demonstrated that HS increases plasma lipopolysaccharide (LPS) concentrations, presumably via disrupting the intestinal barrier function (Hall *et al.* 2001; Pearce *et al.* 2013c).

Collectively, this suggests that changes in energetic metabolism, specifically in insulin homeostasis, might be critical for successfully adapting to and ultimately surviving HS. Our previous attempts to determine insulin sensitivity in HS ruminants and pigs utilizing glucose and insulin tolerance tests are not conclusive (Baumgard and Rhoads 2013). Thus, the objective of the current study was to determine the effects of HS on insulin sensitivity utilizing the hyperinsulinemic euglycemic clamp (HEC): the gold standard technique to determine whole-body insulin action. We hypothesized that HS would increase insulin sensitivity and this altered energetic status would be independent of reduced feed intake.

Materials and Methods

Animals and experimental design

Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals, which took place at the Iowa State University Zumwalt Station Climate Change research facility. Twelve crossbred female pigs (57±4 kg body weight) were randomly assigned to 1 of 2 environmental treatments during 2 experimental periods. During period 1 (5 d in length), all pigs were exposed to thermoneutral conditions (TN; 21.4±0.6°C, 23±3% humidity, 62±1 temperature-humidity index) and fed *ad libitum*. During period 2 (5 d in length), pigs were either exposed to constant HS conditions (31.6±0.4°C, 17±9% humidity, 73±1 temperature-humidity index) and fed *ad libitum* (n = 6), or remained in TN conditions but were pair-fed (PFTN; n = 6) to their HS counterparts, to eliminate the confounding effect of dissimilar nutrient intake. As-fed period 1 daily feed intake (FI) was averaged for each pig and used as a baseline. For each HS pig, the decrease in FI during period 2 was calculated as the percentage of FI reduction relative to period 1 for each day of HS exposure. This percentage of FI reduction was averaged for all the HS pigs per day of exposure and applied individually to the baseline of each PFTN pig. The calculated amount of feed was offered to the PFTN pigs three times daily (~0800, 1400, and 2100 h) in an attempt to minimize post-prandial shifts in metabolism. All pigs were fed a standard industry diet consisting mainly of corn and soybean meal formulated to meet or exceed nutrient requirements (National Research Council 2012). Pigs were individually housed in metabolic crates in 1 of 6 environmental chambers with a 12 h:12 h light-dark cycle. Ambient temperature was controlled but humidity was not governed and both parameters were recorded every 5 min by a data logger (Lascar EL-USB-2-LCD, Erie, PA) in each chamber. Rectal temperature was measured with a digital thermometer (ReliOn, Waukegan, IL),

respiration rate was determined by counting flank movements, and both indices were recorded twice daily (0600 and 2200h) and condensed into daily averages. Body weight was collected at the beginning and the end of the study.

Daily blood sampling

On d 1 of period 1, indwelling catheters were surgically implanted in both jugular veins while pigs were anesthetized (tiletamine/zolazepam, ketamine, and xylazine mixture); using a percutaneous technique as previously described (Sanz Fernandez *et al.* 2014a). From d 4 of period 1, daily blood samples were obtained at 0800 h after a 2 h fast into disposable glass tubes containing 250 U of sodium heparin that were immediately placed on ice. Plasma was harvested by centrifugation at 1300 x *g* and stored at -80°C.

Hyperinsulinemic euglycemic clamp

A HEC was performed after an overnight fast on d 3 of period 1 and d 3 of period 2, in 6 HS pigs and 4 PFTN pigs (catheter dysfunction occurred in 2 PFTN pigs). During the HEC, pigs were constantly infused with 0.6 $mU \cdot min^{-1} \cdot kg\ BW^{-1}$ insulin (expected to be the half maximal effective concentration for the glucose rate of infusion based on (Wray-Cahen *et al.* 2012) at 12 ml/h for 3 h with a syringe pump (NE-300, New Era Pump Systems,Inc., Farmingdale, NY). Porcine insulin (29 U/mg; Sigma-Aldrich, St. Louis, MO) was diluted in 0.1 N HCl to a 290 U/ml stock. Infusates were prepared for individual pigs by mixing the required insulin from the stock with saline containing 4% of each pig's serum. Blood samples were obtained at -60, -45, -30, -15 min relative to the initiation of insulin infusion to determine baseline glucose concentrations for each pig. The euglycemic range was established as ±15% of the mean basal glucose content. An insulin priming infusion was initiated at 0 min at 24 ml/h for 10 min, after which the insulin infusion was decreased to 12 ml/h and this rate was maintained constant

through the end of the HEC. Blood samples (1 ml) were obtained every 5-10 min and immediately analyzed for glucose concentration utilizing a glucometer (McKensson, San Francisco, CA). Exogenous 50% dextrose (VetOne®, MWI Veterinary Supply, Boise, ID) was delivered with a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) and its infusion rate was adjusted in order to maintain euglycemia. Blood samples (3 ml) were collected for further analysis every 15-20 min and immediately placed on ice until plasma harvesting.

Insulin tolerance test

On d 5 of period 2, after an overnight fast, 6 HS and 6 PFTN pigs were anesthetized with the same protocol used for the catheterization surgery. Once anesthetized, subcutaneous adipose tissue (from the cranial dorsum; AT) and skeletal muscle (longissimus dorsi, LD) biopsies were surgically obtained, along with a blood sample. After the initial biopsies, an IV insulin bolus was administered at 0.1 U·kg BW^{-1} (Casu *et al.* 2010). Contralateral biopsies and blood samples were obtained again 15 min after the insulin bolus. Tissue samples were immediately snap frozen and blood samples were placed on ice until plasma harvesting. After the second biopsy, anesthetized pigs were sacrificed by exsanguination.

Blood parameters analyses

Plasma glucose and non-esterified fatty acids (NEFA) concentrations were measured enzymatically using commercially available kits (Wako Chemicals USA, Richmond, VA). The intra- and inter-assay coefficients of variation were 4.5 and 4.6%, and 1.2 and 5.1% for glucose and NEFA, respectively. Plasma insulin and C-peptide concentrations were analyzed using ELISA kits (Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 8.6 and 10.2%, and 8.8 and 4.3% for insulin and C-peptide, respectively.

Western blotting

Whole cell protein from LD and AT was extracted in radioimmunoprecipitation assay (RIPA) buffer containing a protease and phosphatase inhibitor cocktail (Halt™, Thermo Scientific, Rockford, IL). Protein concentrations of the extracts were determined by the bicinchoninic acid assay (BCA, Thermo Scientific, Rockford, IL) and samples were diluted to a common concentration in RIPA buffer without inhibitors. Protein abundance of insulin signaling markers (insulin receptor substrate-1, IRS-1; and protein kinase B, Akt) were assessed using western blot. Samples were loaded (20-40 μg) in 4-20% precast gradient gels (Lonza, Basel, Switzerland) for SDS-PAGE separation using standard techniques. Separated proteins were transferred from the gel to 0.2 μm pore size nitrocellulose membrane (Biorad, Hercules, CA). The membrane was blocked in 5% non-fat dry milk diluted in tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature and incubated in primary antibody (Table 8) overnight at 4°C. Membranes were then washed and incubated in secondary antibody (Table 8) for 1 h at room temperature. For protein detection, membranes were incubated in Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and exposed to film. Bands were quantified using densitometry (Image Lite software 4.0, LI-COR, Lincoln, NE) and relative protein abundance was calculated by normalizing to a common sample included in all gels. Ponceau-S staining of the membrane and GAPDH abundance were used as loading controls.

Calculations and statistical analysis

In order to eliminate the confounding effect of dissimilar baseline values, the plasma metabolites' responses to hyperinsulinemia during the HEC were calculated for each individual pig as the difference between each metabolite's average during the clamped period (i.e. average during hour 2 and 3 of the HEC) and their value during the baseline period (i.e. 1 h prior to the

HEC). The rate of glucose infusion (ROGI) was calculated per pig and per HEC by averaging the ROGI values when plasma glucose content was within the euglycemic range.

All data were statistically analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC). Daily temperature indices, production data, and plasma metabolites were analyzed by repeated measures, using PROC MIXED with an autoregressive covariance structure and day of period 2 as the repeated effect. The model included treatment, day, and their interaction as fixed effects; and period 1 values were used as a covariate. The responses to the HEC and the ITT were analyzed by pre-post repeated measures, using PROC MIXED with an unstructured covariance structure and period or time relative to insulin administration (pre-, post-insulin) as the repeated effects, respectively. The models included treatment, period or time relative to insulin injection, and their interaction as fixed effects.

For each variable in each model, normal distribution of residuals was tested using PROC UNIVARIATE, logarithmic transformation was performed when necessary, and back transformed into the original scale to be reported in the results. Data are reported as least square means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

As expected, during period 2, HS pigs had increased rectal temperatures and respiration rates (1.2°C and ~3 fold, respectively; $P < 0.01$) compared to PFTN controls (Table 9). Overall, HS decreased FI (34%, $P < 0.01$), and by design, PFTN pigs' FI was reduced similarly (Table 9). Feed intake acutely decreased (40%) at the beginning of period 2, reached its minimum on d 3 (due to overnight fasting in preparation for the HEC), and increased thereafter without

recovering to the period 1 FI level ($P < 0.01$; Table 9). By the end of period 2, HS pigs gained more body weight (35%; $P = 0.01$) than PFTN controls (Table 9).

During period 2, daily changes in basal plasma glucose, insulin, and C-peptide did not differ between treatments; however, there was a day effect ($P < 0.05$) in all of these parameters as their concentrations decreased (9, 59, and 44% for glucose, insulin, and C-peptide, respectively) on d 3 as expected because of the overnight fast (Table 10). There was a day effect ($P < 0.01$) on the basal insulin to glucose ratio as it was increased (111%) on d 5 compared to d 1 and 3 of period 2 (Table 10). At the beginning of period 2, basal plasma NEFA acutely increased for both treatments, peaked on d 3, and sharply decreased thereafter ($P < 0.01$); but overall, HS pigs had decreased circulating NEFA (46%; $P < 0.01$) compared to PFTN controls (Table 10).

The baseline glucose concentration prior to the HEC decreased similarly from period 1 to 2 (8%; $P = 0.03$) for both treatments (Table 11). Pre-HEC circulating insulin did not differ between treatments or periods (Table 11). Overall, baseline C-peptide increased (41%; $P < 0.01$) in HS pigs compared to PFTN controls, but this difference was similar in periods 1 and 2 (Table 11). Prior to the HEC, there was a treatment by period interaction in baseline NEFA ($P = 0.04$), as it decreased (33%) in HS pigs from period 1 to 2, while it remained unchanged in PFTN controls (Table 11). During hyperinsulinemia, euglycemia was maintained as the glucose response was close to 0 and did not differ between treatments or periods (Fig. 4A). The overall increase in circulating insulin during the HEC tended to be decreased (43%; $P = 0.10$) in HS pigs compared to PFTN controls, but this difference was similar in periods 1 and 2 (Fig. 4B). As expected, during the HEC, C-peptide and NEFA concentrations decreased, but their response did not differ between treatments or periods (Fig. 4C and D). There was a tendency for a treatment

by period interaction in ROGI (P = 0.10), as it remained similar in HS pigs from period 1 to 2, while the PFTN pig's ROGI decreased (36%; Fig. 4E). The interaction became significant (P = 0.03) when ROGI was normalized to pre-HEC glucose concentration (Fig. 4F).

During the insulin tolerance test (ITT), insulin administration increased circulating insulin (0.026 *vs.* 1.749 ng/mL; $P < 0.01$) and decreased plasma glucose (97 *vs.* 32 mg/dL; $P < 0.01$) concentrations similarly between treatments (data not shown). Overall, HS pigs tended to have increased plasma glucose (59 *vs.* 71 mg/dL; $P = 0.08$) compared to PFTN controls prior and after the insulin injection (data not shown). The ITT increased AT IRS-1 and phospho-Akt protein abundance, as well as the phosphorylated to total Akt ratio (28%, 5 and 5 fold, respectively; $P < 0.01$), similarly between treatments (Fig. 5A, C, and D). Neither treatment nor insulin had an effect on AT Akt abundance (Fig 5B). There was a treatment by time interaction for LD IRS-1 (P = 0.04), as basal protein abundance was increased in HS pigs (42%) compared to PFTN controls, but there were no treatment differences after insulin administration (Fig. 6A). Both LD phospho-IRS-1 and phospho-Akt protein abundance, as well as the phosphorylated to total IRS-1 and Akt ratios increased after the ITT (69%, 61 fold, 62%, 62 fold, respectively; $P < 0.01$), but no treatment differences were detected (Fig. 6B, C, E, F). There were no treatment or insulin effects on LD Akt protein abundance (Fig. 6D).

Discussion

Changes in systemic and intracellular energetic metabolism are crucial for successfully adapting to HS. Specifically, proper insulin homeostasis and insulin action appear critical for acclimation and survival to a severe heat load (Semenza *et al.* 1999; Niu *et al.* 2003; Baumgard and Rhoads 2013). Interestingly, increasing heat shock protein (HSP) abundance improves

insulin sensitivity in various models of diabetes and obesity (Hooper 1999; Kokura *et al.* 2007; Gupte *et al.* 2009), which suggests that insulin signaling and heat adaptation are interrelated. Agriculturally relevant species reared under HS exhibit phenotypic changes, like increased adiposity and reduced milk synthesis (Baumgard and Rhoads 2013), that would not be anticipated based on their energetic status. Insulin is a potent lipogenic and anti-lipolytic signal and thus changes in insulin action may partially explain the body composition differences and milk yield disparity in HS animals.

We have previously reported and corroborated in the current study that, despite marked hypophagia, HS ruminants (Rhoads *et al.* 2009a; Wheelock *et al.* 2010; Baumgard *et al.* 2011) and pigs (Pearce *et al.* 2013a; Sanz Fernandez *et al.* 2014a) do not mobilize as much AT as PFTN counterparts, as demonstrated by a reduction in circulating NEFA and a blunted NEFA response to an epinephrine challenge. A potential explanation for this lack of AT mobilization might be related to changes in insulin homeostasis. We and others have repeatedly reported increased basal insulin in growing and lactating ruminants during HS (Itoh *et al.* 1998; O'Brien *et al.* 2010; Wheelock *et al.* 2010). However, our results in pigs are less conclusive as we have observed increased basal insulin after 7 d of HS (Pearce *et al.* 2013a), no changes in basal insulin but increased circulating C-peptide in early stages of HS (Sanz Fernandez *et al.* 2014a), and no changes in either basal insulin or C-peptide in the current study. Similarly, the insulin response to a glucose tolerance test was consistently increased in ruminants during HS compared to controls, but glucose disposal was either increased (Wheelock *et al.* 2010), unchanged (O'Brien *et al.* 2010), or was blunted (Baumgard *et al.* 2011). In contrast, pigs had a decreased insulin response and blunted glucose disposal following a glucose tolerance test, but coupling both responses (i.e. into an insulinogenic index) suggested that HS animals required less insulin to

stimulate a similar amount of peripheral glucose uptake (Sanz Fernandez *et al.* 2014a). These conflicting results might be due to differences in species (pigs are generally considered more insulin sensitive than ruminants; Brockman and Laarveld 1986), physiological state, experimental design (e.g. constant *vs.* cyclical HS), magnitude and length of the heat load, and timing of the metabolic challenge relative to feeding or peak heat load. Regardless, the glucose tolerance test is not an ideal method to determine insulin action as glucose stimulated insulin secretion, insulin clearance, and peripheral insulin sensitivity all contribute to glucose disposal (Muniyappa *et al.* 2008).

To gain a better appreciation of how HS alters whole-body insulin action we utilized the HEC, which assumes that at the clamped state (i.e. during euglycemia) the amount of exogenous glucose entering the system equals the amount of insulin-stimulated glucose uptake by peripheral tissues (Muniyappa *et al.* 2008). As anticipated, PFTN pigs experienced a 36% decrease in the ROGI from period 1 to 2, which is a well-described homeorhetic adaptation strategy to a reduced plane of nutrition, where insulin sensitivity is decreased in order to spare glucose for tissues that are obligate glucose utilizers (brain, red blood cells; Bauman and Currie 1980). However, HS animals maintained a similar ROGI between periods; in other words, insulin sensitivity remained unchanged during hyperthermia despite the large decrease in FI. This treatment difference in ROGI becomes even more noticeable when considering that baseline glucose prior to the period 2 HEC was numerically decreased in HS pigs compared to period 1 and PFTN pigs. Thus, HS induced an apparent increase (when considering period 1 differences between treatments) in the ROGI compared to PFTN conditions despite being clamped at a numerically lower glucose concentration, and this is why differences become more obvious when normalizing to baseline glucose. The increase in whole-body insulin sensitivity agrees with results in lactating cows

(Skrzypek *et al.* 2010), sheep (Achmadi *et al.* 1993), and the human and rodent literature where thermal therapy improves insulin sensitivity in diabetic and obese individuals (Hooper 1999; Kokura *et al.* 2007; Gupte *et al.* 2009).

Nevertheless, interpreting HEC data is based on certain assumptions. The first assumption is that the induced hyperinsulinemia inhibits hepatic glucose output (Girard 2006) equally between treatments. To our knowledge, there are no reports specifically evaluating insulin's ability to regulate hepatic glucose export during HS. We have previously reported that HS decreases hepatic insulin receptor protein abundance in growing ruminants (O'Brien *et al.* 2008), which might reduce insulin's capacity to inhibit hepatic glucose output. In addition, humans exercising in high ambient temperatures have increased hepatic glucose production (Febbraio 2001) and carbohydrate ingestion fails to inhibit this response (Angus *et al.* 2001). Consequently, insulin may be less effective at reducing hepatic glucose output during HS; a scenario that, if true in the pig, suggests that the treatment differences in insulin mediated whole-body glucose disposal are underestimated and are even greater than the ROGI indicates. The second assumption is that hyperinsulinemia inhibits pancreatic insulin secretion similarly between treatments. This is likely the case in our model as the magnitude of the circulating C-peptide (an indicator of pancreatic insulin secretion; Wallace *et al.* 2004) decrease between treatments did not differ.

The increase in the ROGI during HS indicates increased insulin-induced whole-body glucose uptake; however, the tissues responsible for this increased plasma glucose disposal remain unclear. Skeletal muscle is a likely candidate due to its sheer mass and because it is highly responsive to insulin (Kraegen *et al.* 1985). Although we did not detect differences in basal or stimulated pIRS-1 and phosphorylated to total IRS-1 ratio our molecular data generally

supports this posit, as HS increased muscle basal IRS-1 protein abundance compared to PFTN controls. Additionally, HSP72 overexpression in skeletal muscle prevents the reduction in insulin signaling in response to a high fat diet, and decreases c-jun amino terminal kinase (JNK) activation (a stress kinase responsible for the inactivation of IRS-1; Chung *et al.* 2008). Further, whole-body heat therapy increased glucose uptake and insulin signaling in skeletal muscle, and decreased JNK activation in a HSP72-dependent manner (Gupte *et al.* 2009). Overall, these data indicate that the skeletal muscle is likely a major contributor in glucose usage during HS.

In contrast to the skeletal muscle, we did not observe treatment differences in any of the basal or insulin-stimulated AT insulin signaling markers. This is surprising, as insulin is a potent antilipolytic signal and a likely candidate that may explain the lack of AT mobilization observed in this and other HS experiments (Baumgard and Rhoads 2013; Sanz Fernandez *et al.* 2014a). One explanation may be that, while we evaluated some key components of the insulin signaling pathway, the antilipolytic control during HS is independent of IRS-1 and Akt. Further, the insulin dose may have overwhelmed the insulin signaling pathway (circulating insulin increased ~70 fold), preventing us from detecting subtle differences in activation. Withstanding the absence of differences in AT insulin signaling, the lack of AT mobilization during HS might be the result of enhanced insulin action by other compounds. For instance, plasma lactate, which is increased in a variety of HS models (Baumgard and Rhoads 2013), mediates insulin antilipolytic effects by interacting with the G protein-coupled receptor 81 (Ahmed *et al.* 2010). Similarly, heat-induced increase in circulating prolactin (Alamer 2011) might partially mediate the blunted lipolytic response observed during HS (LaPensee *et al.* 2006; Brandebourg *et al.* 2007). Moreover, the sharp reduction in thyroid hormones observed during HS (Sanz Fernandez *et al.* 2014a) might also contribute to the lack of AT mobilization as thyroid hormones stimulate

lipolysis and NEFA utilization (Pucci *et al.* 2000). Thus, further research is required to establish whether insulin is involved in or governs AT metabolism during HS.

Another plausible fate of glucose disposal might be the immune system. We and others have demonstrated that HS increases plasma LPS (Hall *et al.* 2001; Pearce *et al.* 2013c), presumably due to its deleterious effect on intestinal barrier function and the subsequent increase in intestinal permeability to luminal content (Sanz Fernandez *et al.* 2014b). Interestingly, once activated (e.g. by LPS stimulation) immune cells become obligate glucose utilizers (Maciver *et al.* 2008), and a substantial glucose sink (Greiner *et al.* 1994). Elucidating the immune system's relative contribution to whole-body glucose utilization during HS is of interest.

As mentioned earlier, proper insulin action during HS is critical for survival and adaptation to a heat load as diabetics are more susceptible to heat related-illness/death and insulin administration to diabetic rodents improves survivability to severe HS (Semenza *et al.* 1999; Niu *et al.* 2003). This might be due to insulin's key role in mounting a HSP response and might explain why diabetics have decreased HSP72 expression, correlated with their degree of insulin resistance (Li *et al.* 2006). Interestingly, strategies intended to increase HSP, including thermal therapy, HSP72 overexpression, and HSP co-inducers protect against obesity-induced insulin resistance and improve insulin sensitivity in human and rodent models of diabetes and obesity (Hooper 1999; Kokura *et al.* 2007; Chung *et al.* 2008; Gupte *et al.* 2009). Collectively, these data indicate that there is an interdependent relationship between insulin action and the HSP response, where both are required to successfully adapt to a heat load.

The shift toward glucose utilization observed during HS may also help to explain the importance of increased insulin action in the adaptation to a heat load. For instance, exercising at high temperatures increases skeletal muscle glycogen oxidation at the expense of NEFA (Fink

et al. 1975; Febbraio 2001), and increases the respiratory quotient which suggests enhanced glucose oxidation (Hargreaves *et al.* 1996). The increased reliance in glucose as a fuel during HS might also explain why the liver (a key regulator of plasma glucose) remains responsive to adrenergic signals, while the AT does not (Sanz Fernandez *et al.* 2014a). The mechanism by which HS alters cellular substrate utilization is unknown, but might be related to increased circulating LPS. In skeletal muscle, toll-like receptor 4 activation by LPS favors glucose utilization for ATP production (Frisard *et al.* 2010). However, in contrast to our HS model, LPS signaling typically induces insulin resistance (e.g. decreased glucose uptake) by activating stress kinases (e.g. JNK and inhibitor of kappa B kinase) in thermoneutral conditions (Liang *et al.* 2013). Reasons for the apparent inconsistencies between LPS mediated and HS-induced altered muscle bioenergetics are not clear, but enhanced muscle glycogen utilization or increased non-insulin dependent glucose transport may help explain how glucose's contribution to cellular ATP production increases despite reduced muscle insulin sensitivity. Regardless, determining if a link exists between heat-induced intestinal barrier dysfunction and the increased carbohydrate utilization observed during HS remains of interest.

In the current study, we demonstrated that HS pigs have increased whole-body insulin sensitivity. Further, it appears that the increase in glucose disposal might be partially explained by an increase in skeletal muscle insulin signaling. The mechanism by which HS increases insulin sensitivity and the biological reasons behind an increase in glucose utilization remain unknown. A better understanding of the physiological consequences of HS is critical in order to develop treatment protocols and mitigation strategies for heat-related illnesses.

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Disclosure Statement

The authors are not aware of any affiliation, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

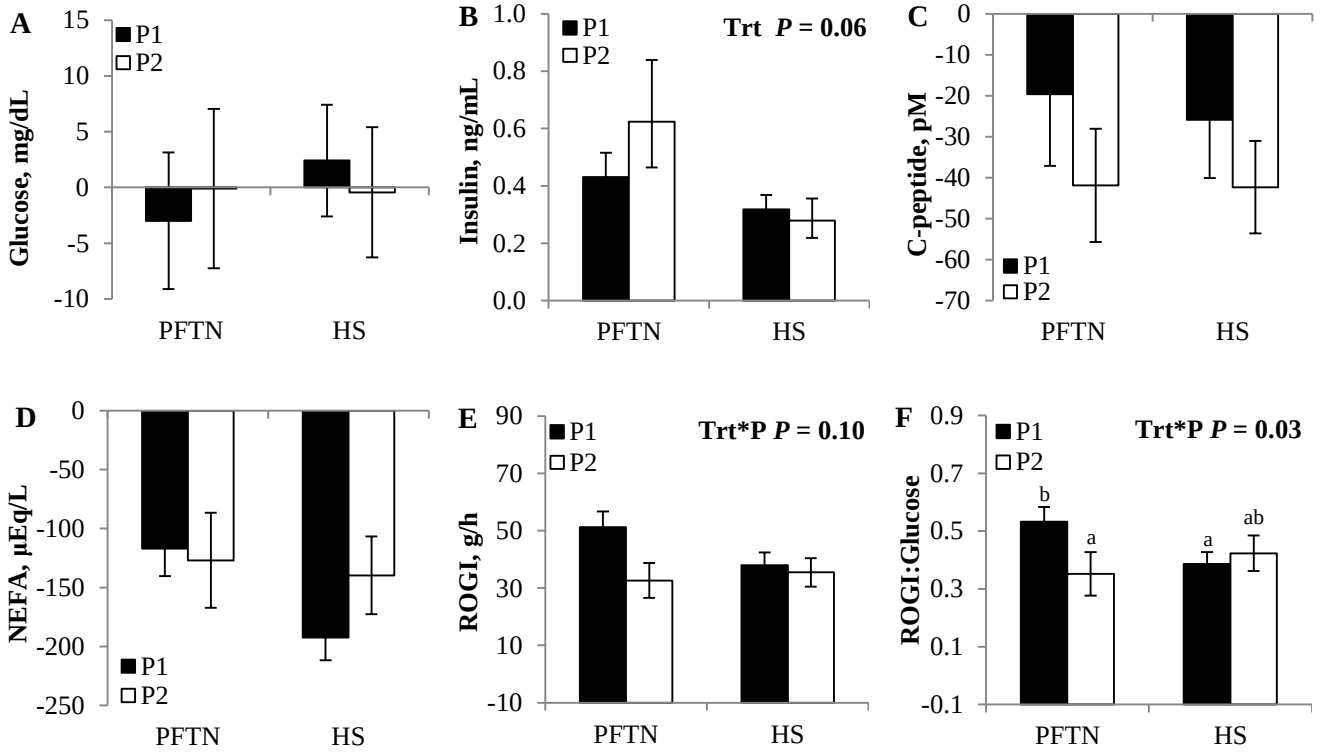

Figure 4. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on plasma (A) glucose, (B) insulin, (C) C-peptide, (D) non-esterified fatty acids (NEFA); (E) rate of glucose infusion (ROGI); and (F) ROGI to euglycemic glucose concentration ratio in response to a hyperinsulinemic euglycemic clamp. On period 1 (P1) all pigs were fed *ad libitum* in thermoneutral conditions. On period 2 (P2) pigs were exposed to either HS or PFTN. [a,b]Means with different letters differ ($P \leq 0.05$)

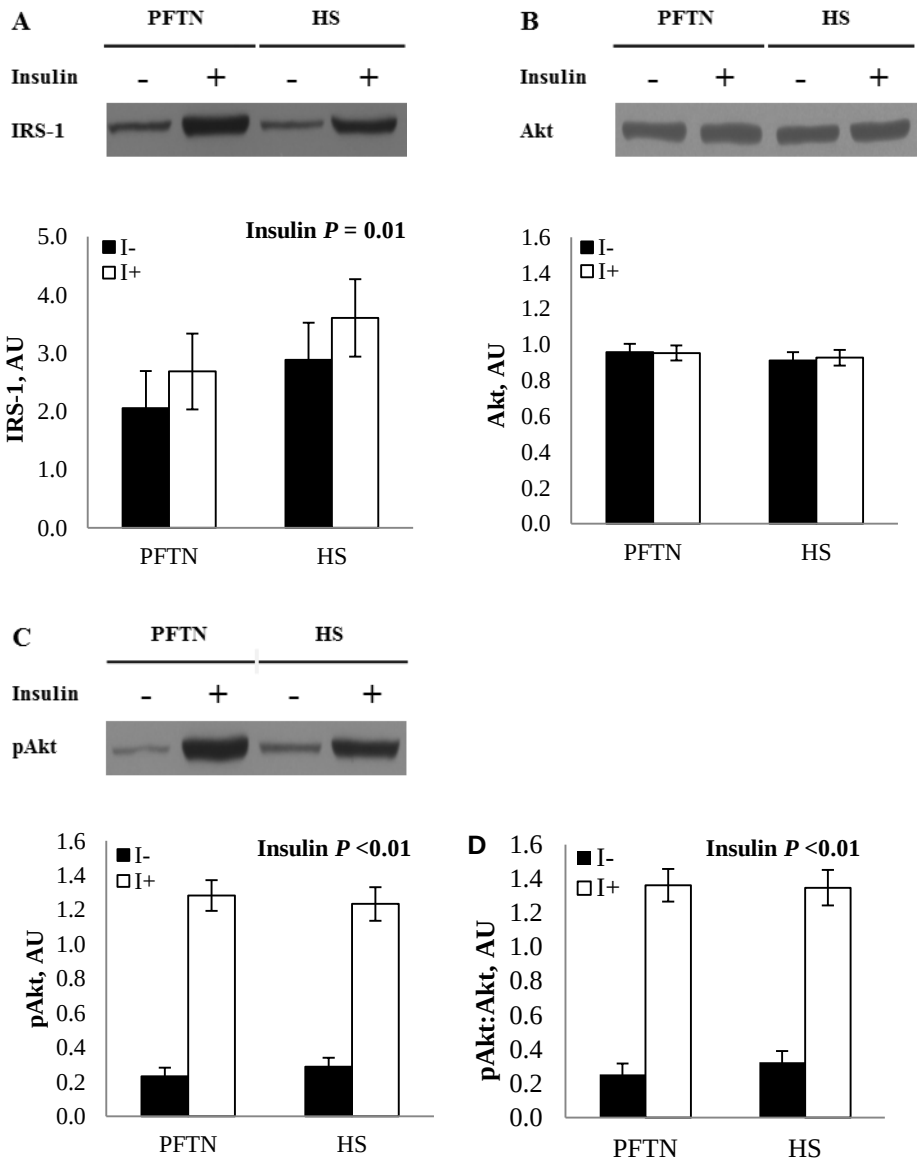

Figure 5. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on the adipose tissue protein abundance of (A) insulin receptor substrate-1 (IRS-1), (B) protein kinase B (Akt), (C) phospho-Ser Akt, and (D) phospho-Ser Akt to total Akt ratio in response to an insulin tolerance test. Tissue biopsies were obtained prior (I-) and 15 minutes after (I+) an intravenous insulin dose (0.1 U·kg BW^{-1}).

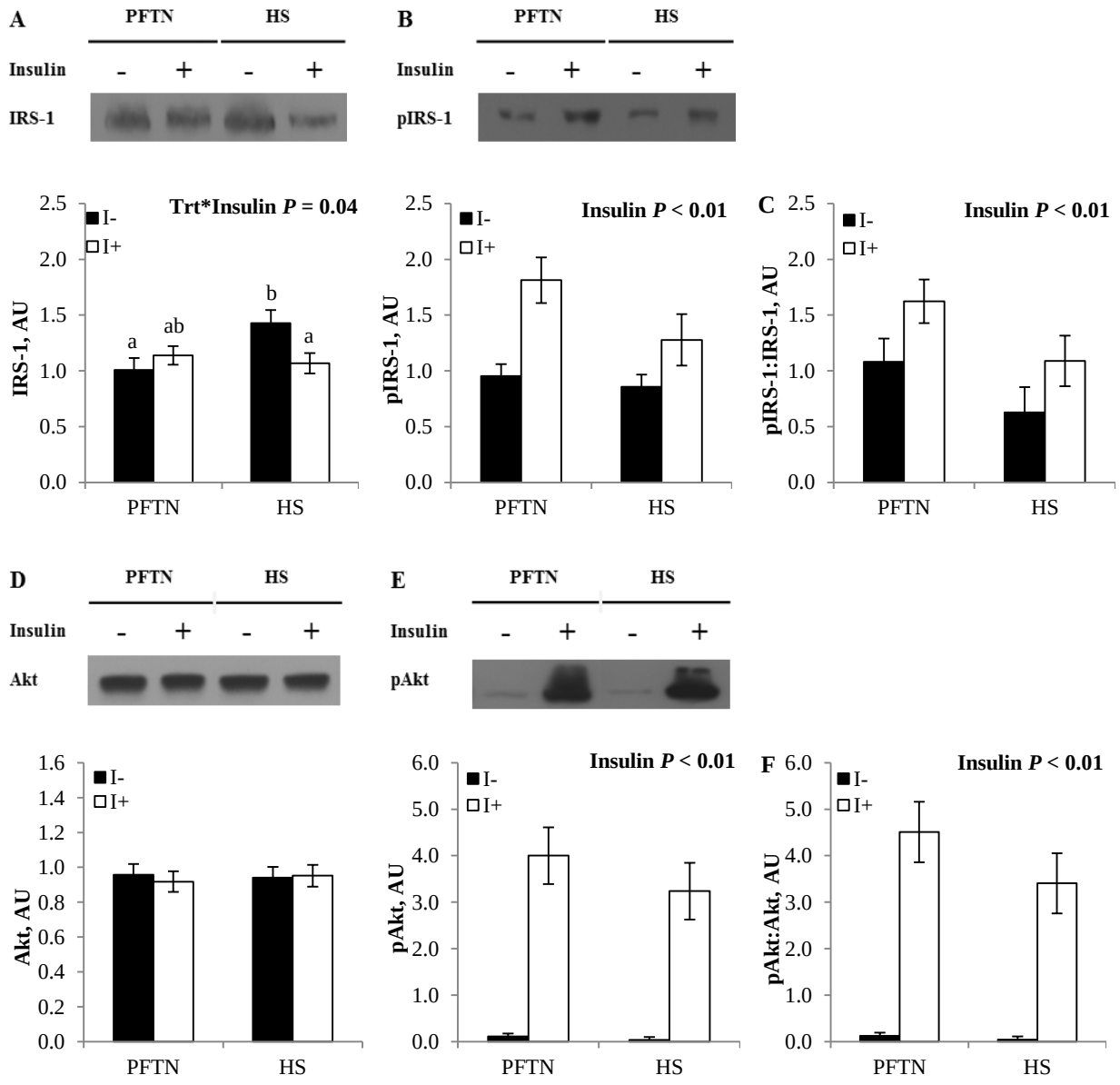

Figure 6. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) and pair-feeding in thermoneutral conditions (PFTN; 20°C) on the skeletal muscle (longissimus dorsi) protein abundance of (A) insulin receptor substrate-1 (IRS-1), (B) phosphor-Tyr IRS-1, (C) phosphor-Tyr to total IRS-1 ratio, (D) protein kinase B (Akt), (E) phospho-Ser Akt, and (F) phospho-Ser to total Akt ratio in response to an insulin tolerance test. Tissue biopsies were obtained prior (I-) and 15 minutes after (I+) an intravenous insulin dose (0.1 U·kg BW^{-1}).

[a,b]Means with different superscripts differ ($P \leq 0.05$).

Table 8. Antibodies and dilutions

Antibody	Dilution	Product no.
IRS-1	1:1,000	Santa Cruz Biotechnology, 7200
Phospho-IRS-1 (Tyr 632)	1:1,000	Abcam, 109543
Akt	1:1,000	Cell signaling Technology, 9272
Phospho-Akt (Ser 473)	1:1,000	Cell signaling Technology, 9271
GAPDH	1:10,000	Santa Cruz Biotechnology, 166545
Goat anti-rabbit IgG HRP	1:50,000	Thermo Scientific, 31462
Horse anti-mouse IgG HRP	1:100,000	Cell signaling Technology, 7076

Table 9. Effects of heat stress on body temperature indices, feed intake and body weight

	Day							*P*		
	P1*	1	2	3	4	5	SEM	Trt[1]	Day	T*D[2]
Rectal temperature, °C:										
PFTN[3]	39.02	39.06^{a}	38.86^{a}	38.82^{a}	38.93^{a}	38.87^{a}	0.16	<0.01	0.61	<0.01
HS[4]	39.09	39.91^{b}	40.23^{cd}	40.33^{d}	40.02^{bc}	40.07^{bc}				
Respiration rate, bpm:										
PFTN	45	40^{xy}	42^{z}	41^{xyz}	34^{x}	39^{yz}	5	<0.01	0.04	0.27
HS	41	93^{xy}	115^{z}	108^{xyz}	95^{x}	114^{yz}				
Feed intake, kg:										
PFTN	1.88	1.10^{y}	1.16^{y}	0.96^{x}	1.47^{z}	1.50^{z}	0.07	0.46	<0.01	0.91
HS	1.89	1.16^{y}	1.19^{y}	0.99^{x}	1.57^{z}	1.48^{z}				
ΔBW[5], kg:										
PFTN						5.2	0.4	0.01		
HS						7.0				

[1]Treatment
[2]Treatment by day interaction
[3]Pair-fed thermoneutral
[4]Heat stress
[5]Change in body weight from period 1 to 2
*Represents period 1 values that were statistically used as covariate
[a-d]Means with different letters differ ($P \leq 0.05$)
[x-z]Days with different letters differ ($P \leq 0.05$)

Table 10. Effects of heat stress on temporal changes in plasma metabolites

	Day							*P*		
	P1*	1	2	3	4	5	SEM	Trt[1]	Day	T*D[2]
Glucose, mg/dL										
PFTN[3]	111.6	95.9^{y}	96.3^{y}	93.3^{x}	99.0^{y}	98.0^{y}	4.3	0.91	0.04	0.42
HS[4]	108.9	100.3^{y}	98.3^{y}	85.1^{x}	103.4^{y}	97.0^{y}	3.6			
NEFA[5], µEq/L										
PFTN	70.6	176.5^{y}	240.4^{y}	253.5^{z}	86.7^{x}	113.0^{x}	27.3	<0.01	<0.01	0.07
HS	71.5	128.1^{y}	73.7^{y}	169.9^{z}	45.4^{x}	56.4^{x}	23.8			
Insulin, ng/mL										
PFTN	0.130	0.061^{y}		0.031^{x}		0.124^{y}	0.017	0.77	<0.01	0.22
HS	0.155	0.088^{y}		0.045^{x}		0.099^{y}	0.015			
C-peptide, pmol/L										
PFTN	113.6	93.1^{y}		61.0^{x}		131.1^{y}	16.7	0.97	<0.01	0.69
HS	159.9	105.0^{y}		63.8^{x}		118.3^{y}	14.3			
Insulin:glucose[6], AU										
PFTN	1.171	0.618^{x}		0.383^{x}		1.312^{y}	0.161	0.71	<0.01	0.27
HS	1.423	0.683^{x}		0.484^{x}		0.979^{y}	0.135			

[1]Treatment
[2]Treatment by day interaction
[3]Pair-fed thermoneutral
[4]Heat stress
[5]Non-esterified fatty acids
[6]Insulin to glucose ratio
*Represents period 1 values that were statistically used as covariate.
[x,y]Days with different letters differ ($P \leq 0.05$)

Table 11. Effects of heat stress on the plasma metabolite baselines prior to a hyperinsulinemic-euglycemic clamp.

	PFTN[1]			HS[2]			*P*		
	P[3]1	P2	SEM	P1	P2	SEM	Trt[4]	P	T*P[5]
Glucose, mg/dL	97	94	5	98	85	4	0.50	0.03	0.11
Insulin, ng/mL	0.035	0.031	0.008	0.051	0.044	0.007	0.12	0.40	0.77
C-peptide, pmol/L	50.9	49.6	6.9	70.7	71.0	5.7	0.01	0.94	0.91
NEFA[6], µEq/L	172.6^{a}	212.5^{ab}	36.3	282.4^{b}	189.3^{a}	29.6	0.31	0.35	0.04

[1]Pair-fed thermoneutral
[2]Heat stress
[3]Period
[4]Treatment
[5]Treatment by period interaction
[6]Non-esterified fatty acids
[a-b]Means with different letters differ ($P \leq 0.05$)

CHAPTER 4: EFFECTS OF SUPPLEMENTAL ZINC AMINO ACID COMPLEX ON GUT INTEGRITY IN HEAT-STRESSED GROWING PIGS

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Abstract

Heat stress (HS) jeopardizes livestock health and productivity and both may in part be mediated by reduced intestinal integrity. Dietary zinc improves a variety of bowel diseases which are characterized by increased intestinal permeability. Study objectives were to evaluate the effects of supplemental zinc amino acid complex (ZnAA) on intestinal integrity in heat-stressed growing pigs. Crossbred gilts (43±6 kg BW) were *ad libitum* fed 1 of 3 diets: 1) control (ZnC; 120 ppm Zn as $ZnSO_4$; n=13), 2) control + 100 ppm Zn as ZnAA (Zn220; containing a total of 220 ppm Zn; n=14), and 3) control + 200 ppm Zn as ZnAA (Zn320; containing a total of 320 ppm Zn; n=16). After 25 day on their respective diets, all pigs were exposed to constant HS conditions (36°C, ~50% humidity) for either 1 or 7 day. At the end of the environmental exposure, pigs were euthanized and blood and intestinal tissues were harvested immediately after

sacrifice. As expected, HS increased rectal temperature ($P \leq 0.01$; 40.23 *v*. 38.93°C) and respiratory rate ($P \leq 0.01$; 113 *v*. 36 bpm). Pigs receiving ZnAA tended to have increased rectal temperature ($P = 0.07$; +0.27°C) compared to ZnC-fed pigs. Heat stress markedly reduced feed intake ($P \leq 0.01$; 59%) and caused BW loss (2.10 kg), but neither variable was affected by dietary treatment. Fresh intestinal segments were assessed *ex vivo* for intestinal integrity. As HS progressed from days 1 to 7, both ileal and colonic transepithelial electrical resistance (TER) decreased ($P \leq 0.05$; 34 and 22%, respectively). This was mirrored by an increase in ileal and colonic permeability to the macromolecule dextran ($P \leq 0.01$; 13 and 56 fold, respectively), and increased colonic lipopolysaccharide permeability ($P \leq 0.05$; 3 fold) with time. There was a quadratic response ($P \leq 0.05$) to increasing ZnAA on ileal TER, as it was improved ($P \leq 0.05$; 56%) in Zn220-fed pigs compared to ZnC. This study demonstrates that HS progressively compromises the intestinal barrier and supplementing ZnAA at the appropriate dose can improve aspects of small intestinal integrity during severe HS.

Keywords: Pigs, Heat stress, Zinc amino acid complex, Intestinal integrity

Implications

Heat stress (HS) jeopardizes animal welfare and profitable pork production during the warm summer months. Environmental hyperthermia compromises the intestinal barrier function resulting in increased permeability to luminal content (bacteria and bacterial components). The leakage of luminal content into the portal and ultimately the systemic circulation might in part mediate the harmful effects of HS on animal agriculture. Identifying nutritional strategies to alleviate the negative impact of HS is critically important. Interestingly, zinc improves gut health in a variety of diseases which are characterized by increased intestinal permeability.

Herein we demonstrate that supplementing zinc amino acid complex partially ameliorates the negative effects of HS on ileal integrity in growing pigs.

Introduction

Heat stress (**HS**) negatively influences animal agriculture and undermines genetic, nutritional, and pharmaceutical advances in feed efficiency. HS-induced economic losses are a result of poor sow performance, reduced and inconsistent growth, decreased carcass quality, and increased veterinary costs (St-Pierre *et al.* 2003; Renaudeau *et al.* 2011). In addition to high ambient temperatures, genetic selection for leaner phenotypes decreases pigs' thermal tolerance, as enhanced protein accretion results in increased basal heat production (Brown-Brandl *et al.* 2003). Therefore, HS is likely one of the primary factors limiting profitable animal protein production and will certainly continue to compromise food security, especially in developing countries (Baumgard and Rhoads 2013). Consequently, identifying nutritional strategies to alleviate the negative impact of HS is critically important.

The deleterious consequences of HS might be partially mediated by its effects on intestinal integrity. For instance, the small intestine is highly sensitive to heat damage (Kregel 2002), and is one of the first tissues up-regulating heat shock proteins during hyperthermia (Flanagan *et al.* 1995). Heat stress increases intestinal permeability (Lambert *et al.* 2002; Pearce *et al.* 2013b) and leads to increasing concentrations of lipopolysaccharide (**LPS**) in portal and systemic blood (Hall *et al.* 2001). Further, endotoxemia is common among heat stroke patients (Leon 2007) and it is thought to play a central role in heat stroke pathophysiology, as survival increases when intestinal bacterial load is reduced (Bynum *et al.* 1979) or when plasma LPS is neutralized (Gathiram *et al.* 1987a).

Zinc is essential for normal intestinal barrier function and the regeneration of damaged gut epithelium (Alam *et al.* 1994). Dietary zinc effectively prevents or improves the loss of intestinal integrity during malnutrition (Rodriguez *et al.* 1996), ethanol-induced intestinal damage (Lambert *et al.* 2003), chronic inflammatory bowel diseases (Sturniolo *et al.* 2001), and infectious diarrhea (Alam *et al.* 1994). Supplemental zinc also reduces intestinal permeability of piglets during weaning (Zhang and Guo 2009).

The objective of the current study was to determine the effects of increasing amounts of zinc amino acid complex (**ZnAA**) supplementation on intestinal integrity in growing pigs exposed to HS. We hypothesized that feeding ZnAA would prevent or ameliorate the deleterious effects of HS on gut permeability.

Materials and Methods

Animals and experimental design

Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Forty-three crossbred gilts (43±6 kg body weight; Pig Improvement Company C22/C29 x L337, Carthage Veterinary Service, Carthage, IL) were blocked by initial body weight (**BW**) and randomly assigned to 1 of 3 diets: 1) control (**ZnC**; containing 120 ppm Zn as $ZnSO_4$; n=13), 2) control + 100 ppm Zn as ZnAA (**Zn220**; containing a total of 220 ppm Zn; n=14), and 3) control + 200 ppm Zn as ZnAA (**Zn320**; containing a total of 320 ppm Zn; n=16). The amount of dietary Zn in the ZnC treatment was selected based upon typical commercial diets for growing pigs in North America. The other supplemental Zn diets represented a 100 and 200 ppm Zn increase relative to the ZnC diet, in order to evaluate the effects of increasing supplemental Zn on intestinal integrity during HS. Other than added ZnAA,

all diets were similar in ingredient and nutrient composition and were formulated to meet or exceed the predicted requirements (National Research Council 1998) for energy, essential amino acids, protein, minerals, and vitamins (Table 12). Zinc amino acid complex was provided from Availa® Zn (Zinpro Corporation, Eden Prairie, MN). Pigs were *ad libitum* fed their respective diets and had free access to water throughout the entire experiment. The study was divided into three experimental periods (**P**): P0, P1 and P2. During P0 (20 ± 1 day in length), pigs were housed in groups according to their dietary treatment for prophylactic enrichment with Zn. At the beginning of P1 (5±1 day in length), pigs were moved into individual pens (57 x 221 cm) and kept in thermal-neutral conditions (19°C; ~61% humidity; temperature-humidity index ≈ 64; Federation of Animal Sciences Societies 2010) for baseline body temperature indices and production parameters collection. During P2, pigs were exposed to constant HS conditions (36°C; ~50% humidity; temperature-humidity index ≈ 85.5) for either 1 or 7 days to evaluate the effects of acute and chronic HS, respectively. Pigs were sacrificed at the end of the environmental exposure using the captive bolt technique followed by exsanguination. A total of 5 pigs were culled from the study: 1 due to illness (ZnC day 1) and 4 due to excessive (>40.95°C) hyperthermia (2 from ZnC d7, 1 from Zn220 day 1, and 1 from Zn220 d7). Data from these pigs were not included in the final analysis. Due to logistic constrictions with the Ussing chambers, 4 replicates were necessary to complete the study with 2 animals per dietary treatment and day of sacrifice per replicate; except for those replicates where animals were culled. Within a replicate, the variables measured and their timing relative to HS initiation were similar.

During P0, environmental conditions were not tightly controlled, but were within the pigs' thermal-neutral zone (Federation of Animal Sciences Societies, 2010). Once in the

environmental rooms (P1 and P2), temperature was controlled but humidity was not governed, and both parameters were monitored and recorded every 30 minutes by a data logger (Lascar EL-USB-2-LCD, Erie, PA). Temperature-humidity index ranged between 62.5-65.5 and 84.0-86.5 during P1 and P2, respectively.

During both P1 and P2, body temperature indices (respiration rate and rectal temperature) were obtained four times a day (0800, 1200, 1600 and 2000 h) and condensed into daily averages. For P1, measurements were further condensed into a single average represented as day -1. Respiration rates (**RR**) were determined by counting flank movements and rectal temperatures (**Tr**) were measured using a digital thermometer (ReliOn, Waukegan, IL). Individual feed intake (**FI**) was recorded daily as-fed during P1 and P2. Body weights were obtained at the initiation of each experimental period, and immediately prior to sacrifice.

Blood was obtained (BD® vacutainers, Franklin Lakes, NJ; containing 12 mg of K_3EDTA) at sacrifice and kept in ice until processing. Plasma was harvested by centrifugation at 1300 x *g* and stored at -80°C for later analysis. Whole sections from both the proximal ileum (1.5 m proximal to the ileal-cecal junction) and distal colon (0.5 m proximal to the rectum) were harvested immediately following euthanasia. Intestinal segments were flushed of luminal contents, and placed immediately into Krebs-Henseleit buffer (**KHBB**; containing 25 mM $NaHCO_3$, 120 mM NaCl, 1 mM $MgSO_4$, 6.3 mM KCl, 2 mM CaCl, and 0.2 mM NaH_2PO_4; pH 7.4) under constant aeration, and transported to the laboratory for mounting into modified Ussing chambers as previously described (Pearce *et al.* 2013b). In addition, ileal sections were fixed in 10% formalin for histological analysis.

Ussing chambers

Ileal and colonic segments of each animal were mounted into modified Ussing chambers (Physiological Instruments, San Diego, CA) for determination of intestinal integrity and active nutrient transport as described by Pearce and colleagues (2013b). Briefly, intestinal samples were placed into the chambers, connected to dual channel current and voltage electrodes. Both the mucosal and serosal sides of the tissue were bathed in KHBB and provided with a constant O_2-CO_2 mixture. Individual segments were then voltage clamped at 0 mV, and transepithelial electrical resistance (**TER**) was determined. After 30 min of stabilization, ileal nutrient transport was measured for glucose, lysine, glutamine, and methionine. Additionally, intestinal segments were tested for permeability to the macromolecule fluorescein isothiocyanate labeled dextran (**FITC-Dextran**; 4.4 kDa; Sigma®, St. Louis, MO) and FITC labeled lipopolysaccharide (**FITC-LPS**, Sigma®, St. Louis, MO); and apparent permeability coefficients (**APP**) were calculated as follows (Pearce *et al.* 2013b):

$$APP = dQ/ (dt \times A \times C_0)$$

Where: dQ/dt = transport rate (µg/min); C_0 = initial concentration in the donor chamber (µg/mL); A = area of the membrane (cm^2).

Histology

Whole ileal samples were fixed in formalin for 24 h and then transferred into 70% ethanol. Fixed samples were referred to the Iowa State University Veterinary Diagnostic Laboratory for sectioning and hematoxylin and eosin staining. Ten intact villi per pig were imaged using Q-capture Pro 6.0 software (Qimaging®, Surey, BC), and each villus height and crypt depth was measured using Image-Pro Plus 7.0 (Media Cybernetics®, Bethesda, MD). Finally, an average height and depth was calculated per pig.

Blood parameters analyses

Plasma glucose concentrations were measured enzymatically using a commercially available kit (Autokit Glucose C2; Wako Chemicals USA, Richmond, VA). Lipopolysaccharide binding protein (**LBP**) concentrations were determined using an ELISA kit (Hycult® biotech, Plymouth Meeting, PA). The intra- and inter-assay coefficients of variation were 2.8 and 3.8, and 12.7 and 4.9% for glucose and LBP, respectively.

Statistical analyses

All data were statistically analyzed using SAS version 9.2 (SAS Institute Inc., Cary, NC). Single measurements were analyzed using PROC GLM, and PROC MIXED was utilized to test daily observations (Tr, RR and FI) by repeated measures with an auto regressive covariance structure and day as the repeated effect. The models evaluated day of sacrifice both independently and together, and included replicate, treatment, day, and treatment x day interaction as fixed effects. Orthogonal contrasts to test for linear and quadratic effects of dietary treatment were performed. When available, the initial value (value during P1) of the parameter of interest was used as a covariate. Data are reported as LSmeans and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

As expected, there were no treatment differences in P1 body temperature indices (Fig. 7). During P2, Tr and RR markedly increased in all treatments ($P < 0.01$; an average of 1.3 °C and 3 fold, respectively; Fig. 7A and B) relative to P1. Both Zn220 and Zn320-fed pigs tended to have increased average Tr ($P = 0.07$; +0.27 °C; Fig. 7A) compared to ZnC-fed pigs.

During P1, FI did not differ among treatments (2.74 kg/day). During P2, HS-induced FI reduction averaged 59%, and the response was not different among treatments (Fig. 8A). From d2, FI increased ($P < 0.05$; +0.40 kg) until plateauing on d4 (Fig. 8A). During P1, there was a quadratic effect ($P \leq 0.05$) on average daily gain as Zn220-fed pigs outgained ZnC and Zn320-fed pigs (1.3 *v.* 1.2 kg/day; data not shown). Body weight did not differ among treatments (data not shown) at the initiation of P1 (70.3 kg) and P2 (78.8 kg). Pigs in all treatments lost a similar amount of BW independently of day of HS (2.1 kg; Fig. 8B).

Irrespective of treatment, ileal TER decreased from days 1 to 7 ($P \leq 0.01$; 33% on average). Feeding ZnAA quadratically improved ileal TER ($P \leq 0.05$) as Zn220-fed pigs had an increased ileal resistance ($P \leq 0.05$; 56%) compared to ZnC, while Zn320-fed pigs did not differ from the other two treatments (Fig. 9). Colonic TER also decreased ($P \leq 0.05$; 22%) as heat exposure progressed, but no differences were observed among dietary treatments (Table 2). Both ileal and colonic FITC-Dextran APP increased ($P \leq 0.01$; 13 and 56 fold, respectively), and colonic FITC-LPS APP tended to increase ($P = 0.07$; 3 fold) from days 1 to 7 of HS; but no dietary treatment differences were detected (Table 13). Overall, there were no treatment effects on ileal glucose, lysine, glutamine or methionine transport; nor did these parameters change from days 1 to 7 (Table 13). However, when analyzing day of sacrifice separately, day 1 glucose transport linearly decreased ($P \leq 0.05$) with increasing levels of ZnAA (24.11, 12.30, 7.42 $\mu A/cm^2$ for ZnC, Zn220 and Zn320, respectively) as Zn320-fed pigs tended to have decreased transport ($P = 0.07$; 69%) compared to ZnC. Irrespective of sacrifice day, no differences in small intestinal architecture (villi height or crypt depth) were observed among treatments (Table 13).

Overall, circulating LBP concentration did not differ among dietary treatments (Table 2). When analyzing day 1 separately, LBP tended to increase linearly (P = 0.06) with increasing levels of ZnAA (13104, 17240, 23561 ng/ml for ZnC, Zn220 and Zn320, respectively). Plasma glucose decreased from days 1 to 7 of HS ($P \leq 0.05$; 134 *v.* 122 mg/dl), but did not differ between dietary treatments (Table 13).

Discussion

Despite advances in heat abatement technologies, HS still compromises animal welfare and reduces productivity during the warm summer months. Genetic selection for thermal tolerance is one potential strategy to mitigate the effects of HS, but this is a long-term solution, and is almost always accompanied by reduced productivity during thermal-neutral conditions (Baumgard and Rhoads 2013). Identifying flexible management approaches to immediately decrease HS susceptibility without negatively influencing traditional production traits would be of great value to global animal agriculture. Dietary supplementation is an example of an easily adjustable tactic that could be utilized by a variety of animal industries and is amenable to diverse production systems.

The consequences of HS on animal productivity might be partially mediated by reduced intestinal integrity (Baumgard and Rhoads 2013). Dietary zinc is required for normal intestinal barrier function (Alam *et al.* 1994), and supplemental zinc improves integrity characteristics in a variety of experimental models and human bowel diseases (Alam *et al.* 1994; Rodriguez *et al.* 1996; Sturniolo *et al.* 2001; Lambert *et al.* 2003; Zhang and Guo 2009). The aforementioned beneficial effects of dietary zinc on bowel pathologies led us to hypothesize that supplemental dietary zinc would alleviate the decrease in intestinal integrity observed in pigs during HS.

In the present study, pigs were exposed to severe constant HS conditions, resulting in a marked increase in all body temperature indices. Interestingly, pigs receiving ZnAA tended to have a slightly elevated Tr (+0.27°C) compared to ZnC-fed pigs and this agrees with zinc's effects in an endotoxemia model (Roberts et al., 2002). This is surprising, as ZnAA-supplemented pigs had similar FI and BW, which are two key variables associated with basal heat production. Respiratory rate was also sharply elevated (3 fold) during heat exposure, but did not differ among treatments. Regardless, both Tr and RR indicate that animals were severely heat-stressed, and understanding why ZnAA-supplemented pigs had a slightly elevated Tr and whether or not this is of biological significance is of interest.

As expected, HS caused an immediate and similar decrease in FI in all dietary treatments. The magnitude of reduction and temporal pattern of nutrient intake is comparable to those observed in a recent experiment by our group (Pearce *et al.* 2013a). Feed intake reduction during HS is a highly conserved response among species and presumably represents an attempt to decrease metabolic heat production (Collin *et al.* 2001; Baumgard and Rhoads 2012). Heat-induced decreased nutrient intake was traditionally assumed to be the reason for reduced weight gain during HS (Collin *et al.* 2001), but we have recently demonstrated that HS causes a variety of metabolic changes independent of nutrient intake (Baumgard and Rhoads 2013), and pigs actually gain more BW during HS than pair-fed thermal-neutral controls (Pearce *et al.* 2013a). In the current experiment, pigs lost ~2.5 kg of BW within the first 24h and had a total weight loss of ~1.5 kg by d7 of HS. The improvement in both FI and BW variables (Fig. 8) as HS progressed implies that pigs were acclimating to their environment. Interestingly, almost all aspects of intestinal integrity deteriorated from days 1 to 7 of HS (Table 13) and this suggests

that acclimation (from a production perspective) is partially independent of HS-induced intestinal barrier dysfunction.

Heat-stressed animals divert blood flow from the viscera to the periphery in an attempt to maximize heat dissipation (Lambert *et al.* 2002), which in addition to hyperthermia leads to intestinal hypoxia (Hall *et al.* 1999). Enterocytes are particularly sensitive to hypoxia and nutrient restriction (Rollwagen *et al.* 2006), resulting in ATP depletion, and increased oxidative and nitrosative stress (Hall *et al.* 2001). This contributes to tight junction dysfunction, and gross morphological changes that ultimately reduce intestinal barrier function (Lambert *et al.* 2002; Pearce *et al.* 2013b). Consequently, HS increases portal and systemic blood LPS concentration (Hall *et al.* 2001; Pearce *et al.* 2013b), which is a potent immunogenic signal that might mediate some of the negative effects of HS on animal production (Baumgard and Rhoads 2013).

In the current study, small intestine integrity parameters (permeability and resistance) markedly deteriorated with progressive heat exposure. As we hypothesized, supplementing ZnAA improved both *ex vivo* measures of integrity and this was especially evident on d7 as Zn220-fed pigs had 66 and 58% improvement in ileal permeability and electrical resistance (Fig. 9). Reasons why intestinal barrier function was not further improved in the Zn320-fed pigs is not clear, but there appears to be a breakpoint where excess dietary Zn is not beneficial and it may in fact have negative consequences (Fujimura *et al.* 2012). Similar to the ileum, colonic permeability and TER deteriorated from days 1 to 7 of HS. However, in contrast, aspects of colon integrity were not ameliorated with supplemental ZnAA. Zinc is primarily absorbed in the small intestine and little or none is taken up by colonocytes (Krebs 2000) and this site of absorption difference might help explain ZnAA's regional effectiveness. Regardless, reasons why supplemental ZnAA improved ileal and not colonic integrity are not obvious, but

understanding the interaction between ZnAA mechanisms and colon physiology remain of interest.

Lipopolysaccharide binding protein is an acute phase protein that binds LPS and mediates its interaction with toll-like receptor 4 (Lu *et al.* 2008), resulting in the activation of the innate immune response. Interestingly, high circulating concentrations of LBP inhibit LPS-induced inflammation (Hamann *et al.* 2005). Once activated, immune cells become obligate glucose utilizers (Maciver *et al.* 2008), which might trigger a whole body shift in nutrient partitioning in order to spare glucose for the immune system (Baumgard and Rhoads 2013). The gut contributes to the homeorhetic response and the increased ileal glucose transport observed during HS in poultry (Garriga *et al.* 2006) and pigs (Pearce *et al.* 2013b) might be a coordinated consequence of increased glucose demand by the activated immune system. Although not affected by dietary treatments overall, circulatory LBP and ileal glucose uptake reciprocally changed after 1 day of HS, as LBP tended to be linearly increased and glucose transport decreased with increasing ZnAA dose (when analyzing day 1 separately). Both parameters can be difficult to interpret but it is plausible that ZnAA increased LBP levels, which attenuated LPS-induced immune system activation and thus its glucose utilization. If this was the case, there would be less need for up-regulating intestinal glucose transport.

The mechanisms by which zinc improves intestinal integrity are not well-understood. Zinc supplementation prevented tight junction opening in a rat colitis model (Sturniolo *et al.* 2002) and reduced intestinal permeability, while increasing the concentration and expression of tight junction proteins in weaning piglets (Zhang and Guo 2009). *In vitro*, zinc supplementation increased TER in Caco-2 cells (Wang *et al.* 2013). In agreement, Caco-2 cells grown in zinc-deficient media had decreased TER, and reduced and delocalized tight junction proteins

(Finamore *et al.* 2008). Zinc supplementation also induces metallothioneins expression in Caco-2 cells (Wang *et al.* 2013), which might act as antioxidants due to their capacity to sequester reactive oxygen species and nitrogen intermediates (Waeytens *et al.* 2009). In addition, zinc increased the expression and concentration of antimicrobial substances like β-defensins in IPEC-J2 cells (Mao *et al.* 2013). Consequently, there appear to be a variety of mechanisms by which dietary zinc can reduce gut "leakiness".

In the aforementioned literature, the amount of supplemental zinc was highly variable ranging from 0.3 mg to 762 mg per day. In the current study and prior to HS, Zn220 and Zn320-fed pigs were receiving approximately 274 and 548 mg per day of Zn from ZnAA, respectively; in addition to the 329 mg of Zn as $ZnSO_4$ present in the control diet. However, differences in model (*in vivo v. in vitro*), species, zinc source, supplementation duration, physiological state, and the type of intestinal insult make it difficult to compare and contrast Zn doses across studies.

Our primary objective was to determine if increasing levels of dietary ZnAA affected intestinal barrier integrity during HS. The current experimental design prevents us from discriminating between the effects of zinc source. Future research to determine differences between ZnAA and inorganic zinc supplementation is warranted. In addition, pigs were exposed to severe HS, and these extreme thermal conditions may have partially blunted the potential benefits of ZnAA. Whether or not milder and cyclical HS (more typical of commercial conditions), would allow for further improvement is of obvious interest. Herein we demonstrate that supplementing ZnAA is effective in partially alleviating the negative effects of severe HS on ileal integrity. Further research is needed to determine whether ZnAA-supplementation benefits at the intestine are translated into production improvements.

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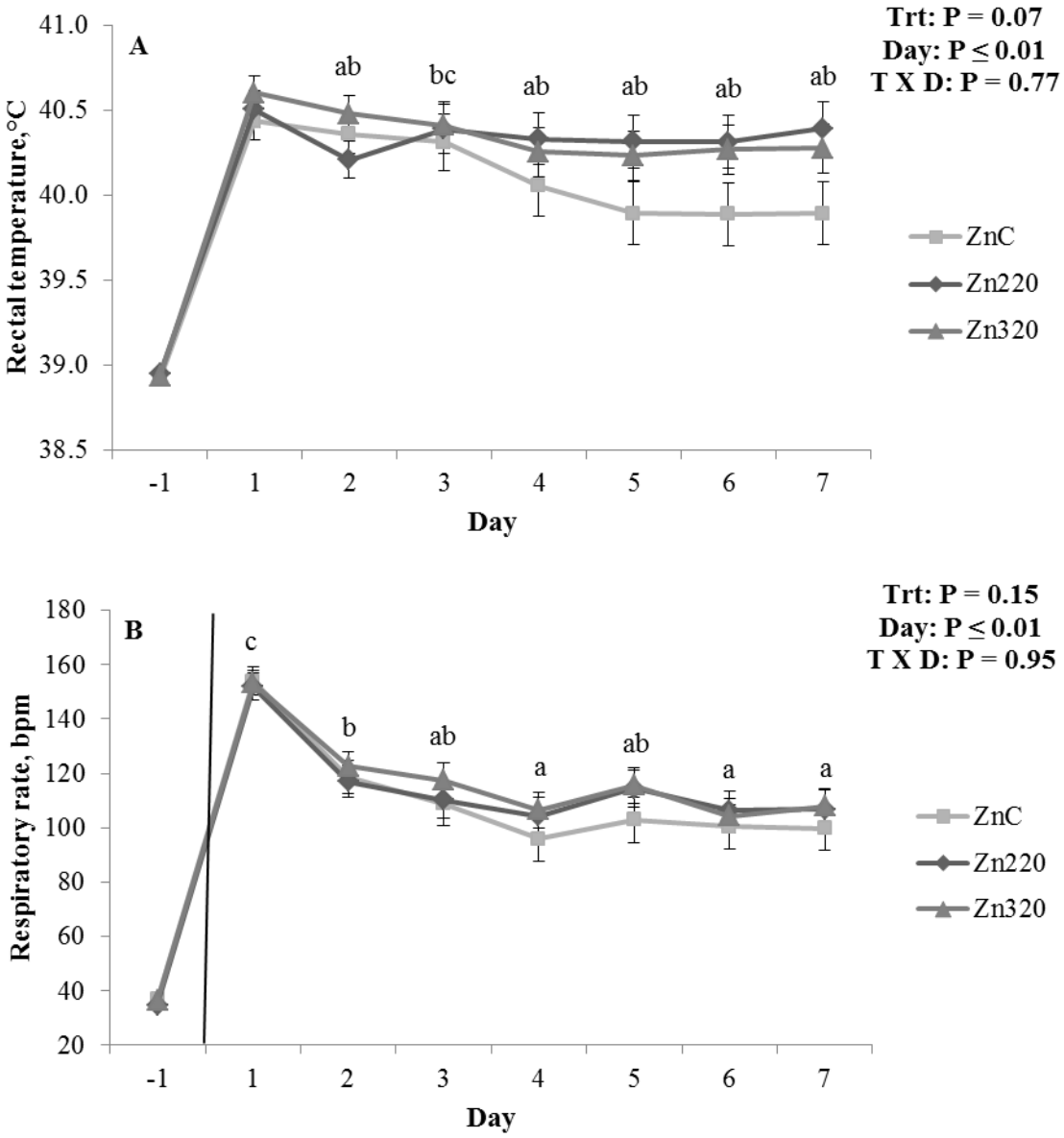

Figure 7. Effects of feeding 120ppm Zn from $ZnSO_4$ (ZnC), 120ppm Zn from $ZnSO_4$ + 100ppm Zn from Zn amino acid complex (Zn220), and 120ppm Zn from $ZnSO_4$ + 200ppm Zn from Zn amino acid complex (Zn320) on (A) rectal temperature and (B) respiratory rates in growing pigs exposed to thermal-neutral conditions (Day -1; Period 1; 19°C) and heat stress conditions (Day 1-7; Period 2; 36°C). Vertical lines indicate the transition from period 1 to 2. ^{a,b,c}Represent differences between days of heat stress ($P \leq 0.05$).

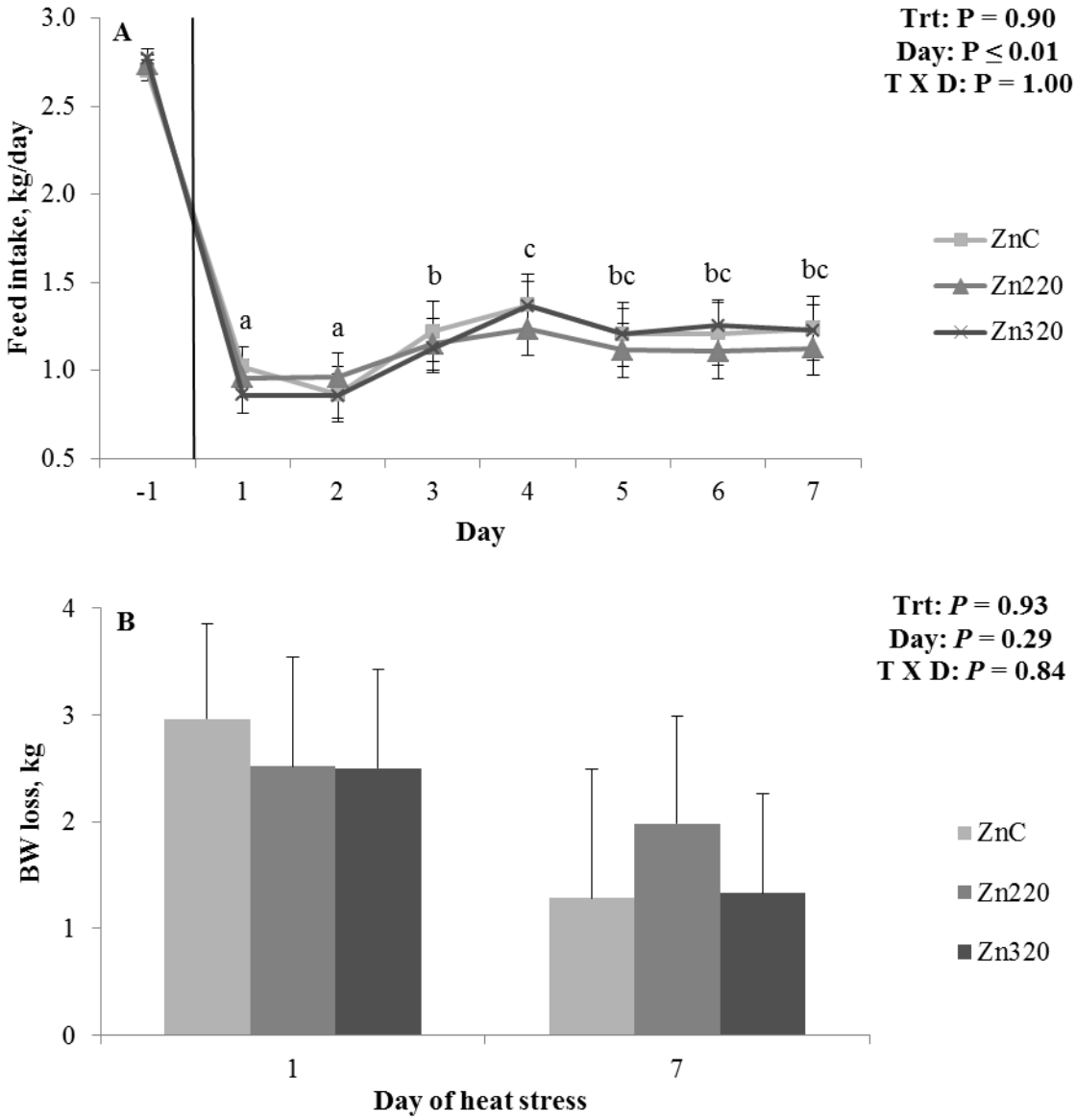

Figure 8. Effects of feeding 120ppm Zn from $ZnSO_4$ (ZnC), 120ppm Zn from $ZnSO_4$ + 100ppm Zn from Zn amino acid complex (Zn220), and 120ppm Zn from $ZnSO_4$ + 200ppm Zn from Zn amino acid complex (Zn320) on (A) feed intake in thermal-neutral conditions (Day -1; Period 1; 19°C) and heat stress conditions (Day 1-7; Period 2; 36°C); and (B) body weight loss after 1 or 7 days of heat stress in growing pigs. Vertical line indicates the transition from period 1 to 2. [a,b,c]Represent differences between days of heat stress ($P \leq 0.05$).

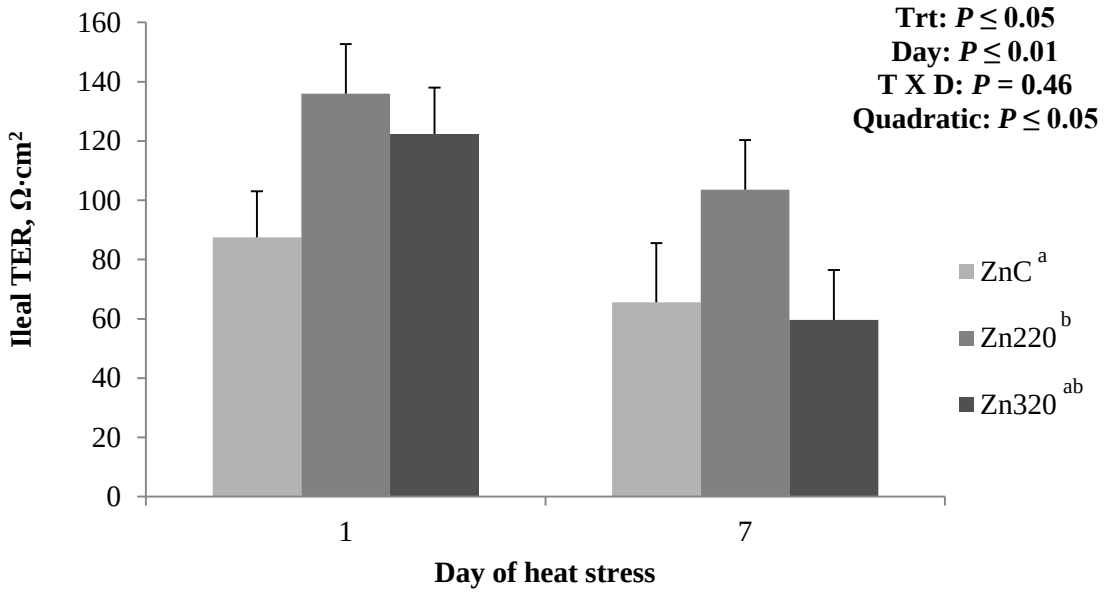

Figure 9. Effects of feeding 120ppm Zn from $ZnSO_4$ (ZnC), 120ppm Zn from $ZnSO_4$ + 100ppm Zn from Zn amino acid complex (Zn220), and 120ppm Zn from $ZnSO_4$ + 200ppm Zn from Zn amino acid complex (Zn320) on ileal transepithelial electrical resistance (TER). [a,b]Represent differences between dietary treatment ($P \leq 0.05$).

Table 12. Ingredients and formulated dietary nutrients

	Diet		
Parameter	ZnC	Zn220	Zn320
Ingredients (%)			
Corn	61.83	61.80	61.75
Soybean meal (46.5)	31.22	31.22	31.22
Soybean oil	2.65	2.65	2.65
l-lysine HCl	0.38	0.38	0.38
dl-methionine	0.18	0.18	0.18
l-threonine	0.17	0.17	0.17
Monocalcium phosphate	1.67	1.67	1.67
Limestone	1.03	1.03	1.03
Salt	0.50	0.50	0.50
Vitamin Premix[1]	0.25	0.25	0.25
Trace Mineral Premix[2]	0.11	0.11	0.11
Selenium Premix (660 ppm Se)	0.01	0.01	0.01
Availa® Zn[3]	-	0.10	0.20
Nutrients			
ME – kcal/kg	3444	3444	3444
Crude Protein %	20.2	20.2	20.2
SID Lys[4] %	1.25	1,25	1.25
Calcium %	0.8	0.8	0.8
Phos. % - t total	0.73	0.73	0.73
Sodium %	0.22	0.22	0.22
Chlorine %	0.34	0.34	0.34
Zinc (ppm, total added)	120	220	320
Selenium (ppm, added)	0.3	0.3	0.3

[1]Provided the following per kg of diet: vitamin A, 7,656 IU; vitamin D, 875 IU; vitamin E, 62.5 IU; vitamin K, 3.75 mg; riboflavin, 13.75 mg; niacin, 70 mg; pantothenic acid, 33.75 mg; vitamin B12, 62.5 µg.

[2]Provided the following per kg of diet: Fe, 121 mg as ferrous sulfate; Zn, 121 mg as zinc sulfate; Mn, 28.6 mg as manganese sulfate; Cu, 12.1 mg as copper sulfate; I, 0.22 mg as calcium iodate; Se, 0.22 mg as sodium selenite.

[3]Zinpro Corporation, Eden Prairie, MN

[4]Standardized ileal digestive lysine

Table 13. Effects of increasing levels of dietary Zn amino acid complex on intestinal and blood parameters in heat-stressed growing pigs

	D[1]1			D7				*P*			Contrast	
Parameter	ZnC	Zn220	Zn320	ZnC	Zn220	Zn320	SEM	Trt[2]	Day	T X D[3]	Linear	Quadratic
Intestinal parameters:												
Ileum												
FITC-Dextran APP[4], µg/ml/min/cm^2	13.50	9.49	6.89	48.32	16.13	39.40	9.98	0.22	<0.01	0.31	0.44	0.11
Glucose transport, µA/cm^2	24.11	12.05	7.92	14.48	23.36	18.03	6.73	0.61	0.48	0.25	0.35	0.79
Lysine transport, µA/cm^2	8.23	5.47	8.12	9.54	8.38	8.86	2.74	0.75	0.47	0.92	0.89	0.46
Glutamine transport, µA/cm^2	3.30	1.34	0.99	1.68	3.22	3.87	1.34	0.99	0.36	0.27	0.97	0.87
Methionine transport, µA/cm^2	0.66	0.38	0.43	1.21	1.45	1.20	0.46	0.35	0.25	0.36	0.22	0.44
Villous height, µm	403	393	406	402	371	387	25	0.73	0.50	0.91	0.82	0.44
Crypt depth, µm	246	267	258	294	262	252	17	0.67	0.39	0.24	0.39	0.88
Colon												
FITC-Dextran APP, µg/ml/min/cm^2	3.68	1.38	4.44	27.17	24.58	11.31	8.00	0.67	0.01	0.54	0.39	0.84
TER, Ω·cm^2	115	113	116	82	82	105	12	0.45	0.02	0.59	0.31	0.52
FITC-LPS APP[6], µg/ml/min/cm^2	3.67	4.28	2.56	14.27	11.23	7.13	4.16	0.59	0.04	0.78	0.35	0.81
Blood parameters:												
LBP[7], ng/ml	13104	16136	23561	11853	11432	16131	4947	0.28	0.28	0.83	0.15	0.58
Glucose, mg/dl	138	127	138	125	122	119	8	0.64	0.05	0.63	0.68	0.38

ZnC = 120ppm Zn from $ZnSO_4$; Zn220 = ZnC + 100ppm Zn from Zn amino acid complex; Zn320 = ZnC + 200ppm Zn from Zn amino acid complex

[1]Day of heat stress

[2]Treatment

[3]Treatment by day interaction

[4]Fluorescein isothiocyanate labeled dextran apparent permeability coefficient

[5]Transepithelial electrical resistance

[6]Fluorescein isothiocyanate labeled lipopolysaccharide apparent permeability coefficient

[7]Lipopolysaccharide binding protein

CHAPTER 5: EFFECTS OF DAIRY PRODUCTS ON INTESTINAL INTEGRITY IN HEAT-STRESSED PIGS

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Disclosure statement

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Abstract

Heat stress compromises intestinal integrity which may partially explain its negative effects on animal health and productivity. Research suggests that challenged intestinal barrier function improves with dietary dairy products in various models. Thus, the study objective was to evaluate the effects of bovine milk whey protein (WP) and colostral whey protein (CWP) on intestinal integrity in heat-stressed pigs. Crossbred gilts (39±3 kg BW) were fed 1 of 4 diets (n=8 pigs/diet): control (Ct), control diet containing an 80% WP and 20% CWP product (WP80),

control diet containing a 98% WP and 2% CWP product (WP98), and control diet containing a 100% WP product (WP100). After 7d on experimental diets, pigs were exposed to constant heat stress conditions (32°C) for 24h. There were no treatment differences in growth or body temperature indices prior to heat stress. During heat exposure, both rectal temperature and respiration rate increased (+0.85°C and 3 fold, respectively; $P<0.01$), and feed intake and body weight decreased (44% and -0.5kg, respectively; $P<0.01$), but neither variable was affected by dietary treatments. Plasma L-lactate and D-lactate concentrations increased (36%; $P<0.01$) and tended to increase (19%; $P=0.09$) with heat stress. After 24h of heat exposure, WP100-fed pigs had lower plasma D-lactate relative to Ct-fed pigs. Ileal transepithelial electrical resistance was decreased (37%; $P=0.02$) in WP80 pigs, compared to controls. No differences were detected in other intestinal integrity *ex vivo* measurements. These data demonstrate that dietary WP and CWP did not mitigate intestinal integrity dysfunction during severe heat stress.

Keywords: Pigs, Heat stress, Dairy products, Intestinal integrity

Abbreviations

CWP = Colostral whey protein; FITC-Dextran APP = Fluorescein isothiocyanate labeled macromolecule dextran apparent permeability coefficient; HS = Heat stress; LBP = Lipopolysaccharide binding protein; LPS = Lipopolysaccharide; TER = Transepithelial electrical resistance; WP = Milk whey protein

Introduction

Heat stress negatively affects animal agriculture by reducing productivity and jeopardizing animal welfare. For the global swine industry, the effects of HS on growth, carcass quality, health and reproduction undermine efforts to improve efficiency and sustainability (St-Pierre *et al.* 2003). Further, the deleterious effects of HS will be aggravated if climate continues

to warm as predicted (Luber and McGeehin 2008). In addition, genetic selection for rapid skeletal muscle growth might increase pigs' susceptibility to HS, as enhanced lean tissue accretion is accompanied by increased metabolic heat production (Brown-Brandl *et al.* 2003). Therefore, developing nutritional strategies to alleviate the effects of HS would be a key tool to maximize efficient animal protein production during the warm summer months (Rhoads *et al.* 2013).

Heat stress compromises intestinal barrier function in a variety of species (Lambert *et al.* 2002), and this might partially explain its effect on animal production. In agreement, we have repeatedly demonstrated that heat-stressed growing pigs have reduced intestinal integrity and function (Pearce *et al.* 2013b; Pearce *et al.* 2013c; Sanz Fernandez *et al.* 2014b). This is caused by the re-distribution of blood flow to the periphery for increased heat dissipation, which reduces oxygen and nutrient supply to intestinal tissues, resulting in enterocyte damage and increased permeability to luminal content and pathogens (Hall *et al.* 1999). The increased passage of bacterial components (e.g. LPS) and bacteria into portal and ultimately systemic circulation are partially responsible for the pathophysiology of heat related illnesses, as reducing intestinal bacterial load (Bynum *et al.* 1979) or neutralizing plasma LPS (Gathiram *et al.* 1987a) increase heat stroke survival. Hence, approaches attempting to preserve and restore intestinal barrier function might improve animal production and wellbeing during environmentally-induced hyperthermia.

Dietary dairy products have been demonstrated to improve gut health. For instance, milk, colostrum, and whey protein supplementation are beneficial in models of induced intestinal damage in both mice and humans (Playford *et al.* 1999; 2001), as an ulcerative colitis treatment (Khan *et al.* 2002), and *in vitro* in response to a tight junction disruptor (Prosser *et al.* 2004).

Interestingly, dietary dairy products ameliorated the effects of HS on intestinal barrier function in both mice (Prosser *et al.* 2004) and a human colonic cell line (Marchbank *et al.* 2011).

Thus, current study objectives were to determine the effects of dietary bovine WP concentrate and CWP concentrate on intestinal integrity parameters and blood biomarkers of "leaky gut" in heat-stressed pigs. We hypothesized that feeding dairy products would prevent or at least ameliorate the deleterious effects of HS on gut permeability.

Materials and Methods

Animals and experimental design

Colostrum whey protein concentrate was obtained from Sterling Technologies and was blended with conventional WP concentrate (Main Street Ingredients). Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Thirty two crossbred gilts (39±3 kg body weight) were stratified by BW and then randomly assigned to 1 of 4 diets: 1) control (Ct), 2) the control diet containing an 80% WP and 20% CWP test product (WP80); 3) the control diet containing a 98% WP and 2% CWP test product (WP98); and 4) the control diet containing a 100% WP test product (WP100). Test dairy products were similar in nutrient composition (Table 14) and constituted 7% of the respective diets in order to provide 100 g/d of protein. Other than the added test dairy products, all diets were similar in ingredient and nutrient composition and were formulated to meet or exceed the estimated requirements (National Research Council 1998) for essential amino acids, protein, minerals, and vitamins (Table 15). Pigs were *ad libitum* fed their respective diets and had free access to water throughout the entire experiment.

The study began after 3 days of acclimation to individual crates and was divided into two experimental periods. During period 1, pigs remained in constant thermoneutral conditions (19°C; ~46% humidity; temperature-humidity index ≈ 63; Federation of Animal Sciences Societies 2010) for 7 days. During period 2, pigs were exposed to constant HS conditions (32°C; ~26% humidity; temperature-humidity index ≈ 76) for 24 h. At the end of period 2, pigs were sacrificed using the captive bolt technique followed by exsanguination. Throughout the experiment, ambient temperature was controlled but humidity was not governed, and both parameters were monitored and recorded every 30 minutes by data loggers (EL-USB-2-LCD, Lascar). Temperature-humidity index ranged between 58-65 and 75-79 during period 1 and 2, respectively.

During period 1, body temperature indices (respiration rate and rectal temperature) were obtained four times a day (0800, 1200, 1600 and 2000 h) and condensed into daily averages and period average. During period 2, temperature indices were obtained at 0, 4, 8, 12 and 24 h relative to the initiation of HS. Respiration rate was determined by counting flank movements and rectal temperature was measured using a digital thermometer (V901H, Vicks®). Individual feed intake was recorded daily as-fed throughout the experiment. Body weights were collected at the beginning of each period and immediately prior to sacrifice.

Blood was obtained (K_2EDTA blood tubes, BD vacutainers®, cat# 367861) on day 6 of period 1 (24 h prior to initiation of HS) and at sacrifice and kept in ice until processing. Plasma was harvested by centrifugation at 1300 x *g* and stored at -80°C for later analysis. Whole sections from both the proximal ileum (1.5 m proximal to the ileocecal junction) and distal colon (0.5 m proximal to the rectum) were harvested immediately following euthanasia. Intestinal segments were processed as previously described (Sanz Fernandez *et al.* 2014b). Due to

logistical constraints in sample collection and analysis, groups of 4 pigs (1 pig per treatment) were sacrificed twice a day (8 pig/d) for 4 consecutive days. Each group of 4 pigs was considered a "set" for statistical purposes. The timing of each measured variable was similar among sets.

Ex vivo measures of intestinal integrity

Ileal and colonic segments of each animal were mounted into modified Ussing chambers (Physiological Instruments) for determination of intestinal integrity. The TER and FITC-Dextran APP (4.4 kDa; Sigma®, cat# FD4) were measured and calculated as previously described by Pearce et al. (2013b) Chamber slides had a surface area of 0.7 cm^2 and all readings were corrected to a 1 cm^2 surface.

Blood parameters analyses

Plasma L-lactate and D-lactate concentrations were measured enzymatically using commercially available kits (Biomedical Research Service Center). Plasma LBP concentrations were determined using an ELISA kit (Hycult® biotech, cat# HK503). The intra- and inter-assay coefficients of variation were 6.6 and 5.1% for L-lactate, 1.5 and 2.3% for D-lactate, and 8.7 and 22.2% for LBP.

Statistical analyses

Data are reported as LSmeans and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. Variables with single measurements were statistically analyzed using the PROC GLM procedure of SAS version 9.2 (SAS Inst. Inc.). The model included treatment and set as fixed effects. For a given variable, when an initial measurement (at the beginning of the period or during period 1) was available it was used as a covariate.

Variables with multiple measurements were analyzed using the PROC MIXED procedure of SAS. Each animal's respective parameter was analyzed using repeated measures with an auto regressive covariance structure. The repeated effect was hour after initiation of HS (rectal temperature and respiration rate) or period (blood parameters). The model included treatment, the repeated effect, set, and treatment by the repeated effect interaction as fixed effects; and covariate when available. For both procedures, set and the covariate were only kept in the model if their $P \leq 0.20$. Contrasts were performed to estimate differences between each dietary treatment and the Ct-fed pigs. For each variable, residuals distribution was tested for normality and logarithmic transformation was performed when necessary.

Results

During period 1, there were no differences in body temperature indices (Fig. 10) among diets. As expected during period 2, rectal temperature and respiration rate markedly increased by 0.85 °C and ~3 fold, respectively ($P < 0.01$; Fig. 10). There was a time effect ($P \leq 0.05$; Fig. 10) for both indices as rectal temperature and respiration rate peaked at 12 and 4 h post-HS initiation, respectively; however, no dietary treatment differences in these variables were detected. Both rectal temperature and respiration rate remained markedly increased (0.6°C and 3 fold, respectively) compared to period 1 (Fig. 10.A and B).

During period 1, feed intake (2.1 kg/d), average daily gain (0.74 kg/d) and gain to feed ratio (0.35) were not different between diets (Table 16). During period 2 (after 24 h of HS), pigs in all treatments similarly reduced their intake and lost BW (44% and -0.5 kg, respectively; Table 16).

There was a diet effect on ileal TER, as it was decreased ($P \leq 0.05$) and tended to be decreased ($P \leq 0.10$) in WP80 and WP100-fed pigs (37 and 27%, respectively; Table 4), compared to controls. Ileal TER did not differ between WP98 and Ct-fed pigs. There were no dietary treatment differences on ileal FITC-Dextran APP or any measure of colonic integrity (Table 17).

Both plasma L-lactate and D-lactate concentration increased ($P < 0.01$; 36%) and tended to increase ($P = 0.09$; 19%), respectively from period 1 to period 2, but the magnitude of the response was similar among dietary treatments (Fig. 11.A and B). During period 2 and using period 1 as a covariate, plasma D-lactate was decreased in WP100-fed pigs ($P \leq 0.05$; 29%; Fig. 11.C), when compared to Ct-fed pigs. Neither dietary treatment nor period had an effect on plasma LBP concentration (Fig. 11.C).

Discussion

Heat stress negatively affects animal agriculture by reducing growth and reproductive performance and jeopardizing animal welfare. Heat-stressed animals redistribute blood to the periphery in an attempt to maximize radiant heat dissipation (Lambert *et al.* 2002). Subsequent visceral vasoconstriction leads to intestinal hypoxia in addition to hyperthermia (Hall *et al.* 1999; Pearce *et al.* 2013c). As demonstrated by the early up-regulation of heat shock proteins during hyperthermia (Flanagan *et al.* 1995), enterocytes are extremely sensitive to oxygen and nutrient restriction (Rollwagen *et al.* 2006), resulting in ATP depletion, and increased oxidative and nitrosative stress (Hall *et al.* 2001). Ultimately, HS causes marked morphological changes, tight junction disruption, and reduced intestinal barrier function (Lambert *et al.* 2002). Increased intestinal permeability during HS, elevates portal and systemic blood LPS concentration (Hall *et*

al. 2001; Pearce *et al.* 2013b), which might mediate some of the negative effects of HS on animal production (Baumgard and Rhoads 2013). Interestingly, dairy products have improved intestinal barrier function in a variety of models. Further, we have recently demonstrated that dietary supplementation with zinc partially ameliorated the effects of HS on intestinal permeability in pigs (Sanz Fernandez *et al.* 2014b), demonstrating that nutritional management is a feasible mitigation strategy for environmental hyperthermia. Thus, we hypothesized that dietary bovine WP and CWP would alleviate the decrease in intestinal integrity observed in pigs during HS.

Contrary to our hypothesis and in disagreement with the literature, feeding WP/CWP did not improve direct measures (i.e. TER and FITC-Dextran APP) of ileal or colonic permeability. Playford et al. (1999; 2001) demonstrated that dairy product supplementation ameliorates the effects of non-steroidal anti-inflammatory drugs on the gastro-intestinal tract in rodents (i.e. WP and CWP) and humans (i.e. bovine colostrum and milk). In addition, local treatment with CWP improved symptoms and histological scores of ulcerative colitis patients (Khan *et al.* 2002). *In vitro* studies have also shown the beneficial effects of bovine colostrum and goat milk on TER in response to a tight junction disruptor (Prosser *et al.* 2004). Finally, dairy products improved intestinal integrity in heat-stressed rats (Prosser *et al.* 2004) and in an *in vitro* model of hyperthermia (Marchbank *et al.* 2011). The mechanism by which dairy products may protect intestinal health is not fully elucidated. Both bovine WP and CWP are rich in antimicrobial proteins (e.g. glucomacropeptides, lactoferrin), immunoglobulins, growth factors (transforming growth factor-β), and specific amino acids (glutamine, cysteine, and threonine; Krissansen 2007). However, the composition of these products is highly variable depending on the origin (e.g. breed, alimentation and health status), the time of collection, and the post-collection

processing; making difficult to identify the bioactive constituents responsible for their positive effects (Kelly 2003). With regard to intestinal health, several mechanisms of action have been attributed to dairy products, including up-regulation of heat-shock proteins (Marchbank *et al.* 2011) and tight junction proteins (Hering *et al.* 2011), or the increase in mucin production (Sprong *et al.* 2010). Consequently, there appear to be a variety of mechanisms by which dietary dairy products can reduce gut "leakiness".

Reasons for the lack of a protective response to our dietary treatments are not clear. In the present study, pigs were severely heat-stressed (constant 32°C without a thermal recovery period during the night), as demonstrated by a marked increase in all the body temperature indices (rectal temperature = +0.85 °C, respiration rate = +76 breath/min) and sharp decrease in feed intake (44%). We hypothesize that the severe HS may have blunted the potential beneficial effects of WP and CWP on intestinal health. Whether or not mild and/or cyclical HS conditions (more similar to natural heat stress events), would allow for dairy products to express their improvement on intestinal permeability variables remains of interest. Not only were the products in the current study ineffective, feeding WP80 (the diet with the highest content in CWP: 20%) actually increased ileal permeability. This is not unprecedented as it agrees with a human report, where subjects supplemented with bovine colostrum had increased intestinal permeability after a standardized exercise program compared to controls and individuals receiving WP (Buckley *et al.* 2009).

We recognize that our experimental design (with no thermoneutral control treatment) makes it difficult to directly demonstrate that the gut was actually compromised by HS. However, utilizing a similar heat load and type of pigs (i.e. genetics, BW, gender) we have repeatedly reported that direct intestinal measurements of gut integrity deteriorated after 24 h of

thermal exposure (Pearce *et al.* 2013b; Pearce *et al.* 2013c), and circulating LPS increased (Pearce *et al.* 2013d). In addition, in a similar experimental design we have reported an increase in oxidative stress parameters in skeletal muscle, ostensibly the consequence of heat-stressed induced endotoxemia (Rosado Montilla *et al.* 2014). In the current experiment we measured blood biomarkers of leaky gut (i.e. D- and L-lactate and LBP) in both period 1 and 2. D-lactate is a product of microbial metabolism, so its presence in blood likely indicates an increase in gut leakiness (Ewaschuk *et al.* 2005; Nielsen *et al.* 2012). As expected, D-lactate increased from period 1 to period 2, confirming that the intestinal barrier function was compromised after 24 h of HS. Lipopolysaccharide binding protein is an acute phase protein that binds LPS and mediates its interaction with toll-like receptor 4 (Lu *et al.* 2008), resulting in the activation of the innate immune response. Interestingly, high circulating concentrations of LBP inhibit LPS-induced inflammation (Hamann *et al.* 2005). We have previously reported that plasma LBP decreases as HS progresses (1-12 h) and intestinal integrity deteriorates (Pearce *et al.* 2013d). Reasons why we did not observe a period effect on plasma LBP concentrations remain unknown. Noteworthy, at the end of period 2, WP100-fed pigs had lower plasma D-lactate concentration compared to Ct-fed pigs and a numerical increase in plasma LBP from period 1 to period 2, which has been previously associated with decreased circulating LPS (Pearce *et al.* 2013d). In agreement, WP100-fed pigs had a numerical improvement in intestinal integrity (increased TER and decreased FITC-dextran APP) at the colon level, where most of the microflora is located, which might explain the aforementioned changes in plasma parameters.

L-lactate is the product of anaerobic glycolysis. The rationale to utilize this parameter as a biomarker of intestinal integrity is based on the release of intracellular L-lactate by damaged intestinal cells (Nielsen *et al.* 2012). However, a similar phenomenon occurs in non-intestinal

cells, which makes L-lactate a nonspecific measurement of cellular damage. Our baseline (period 1) L-lactate levels are higher than those previously reported (Rixen *et al.* 2002; Nielsen *et al.* 2012), however, our blood collection method (jugular venipuncture), which requires physical constraint, could have triggered a stress response which is known to increase L-lactate levels (Hemsworth *et al.* 2002). Nevertheless, this procedure was consistently performed in both periods. Notably, the increase in plasma L-lactate has been repeatedly observed during HS (Baumgard and Rhoads 2013). Our recent data suggests that complete glucose oxidation in skeletal muscle is decreased during HS, thus L-lactate is produced via aerobic glycolysis (Baumgard and Rhoads 2013). Therefore, increased circulating L-lactate might be the result of metabolic adaptations to HS, resembling the Warburg effect employed by cancerous cells (Kim and Dang 2006). Whether the increase in circulating L-lactate from period 1 to period 2 is a result of cellular damage (presumably intestinal damage) or altered systemic glucose metabolism remains to be elucidated.

Heat stress is one of the largest impediments to efficient animal production. A hallmark of heat-stressed animals is a compromised intestinal integrity, and the subsequent endotoxemia might contribute to reduced animal productivity. We have herein demonstrated that feeding a combination of bovine WP and CWP in the tested proportions failed to ameliorate the effects of severe and constant HS on intestinal integrity of pigs. Whether milder and cyclical HS conditions (resembling those in commercial settings) and different levels of inclusion of the test dairy products would allow for improvement on intestinal health remains of interest.

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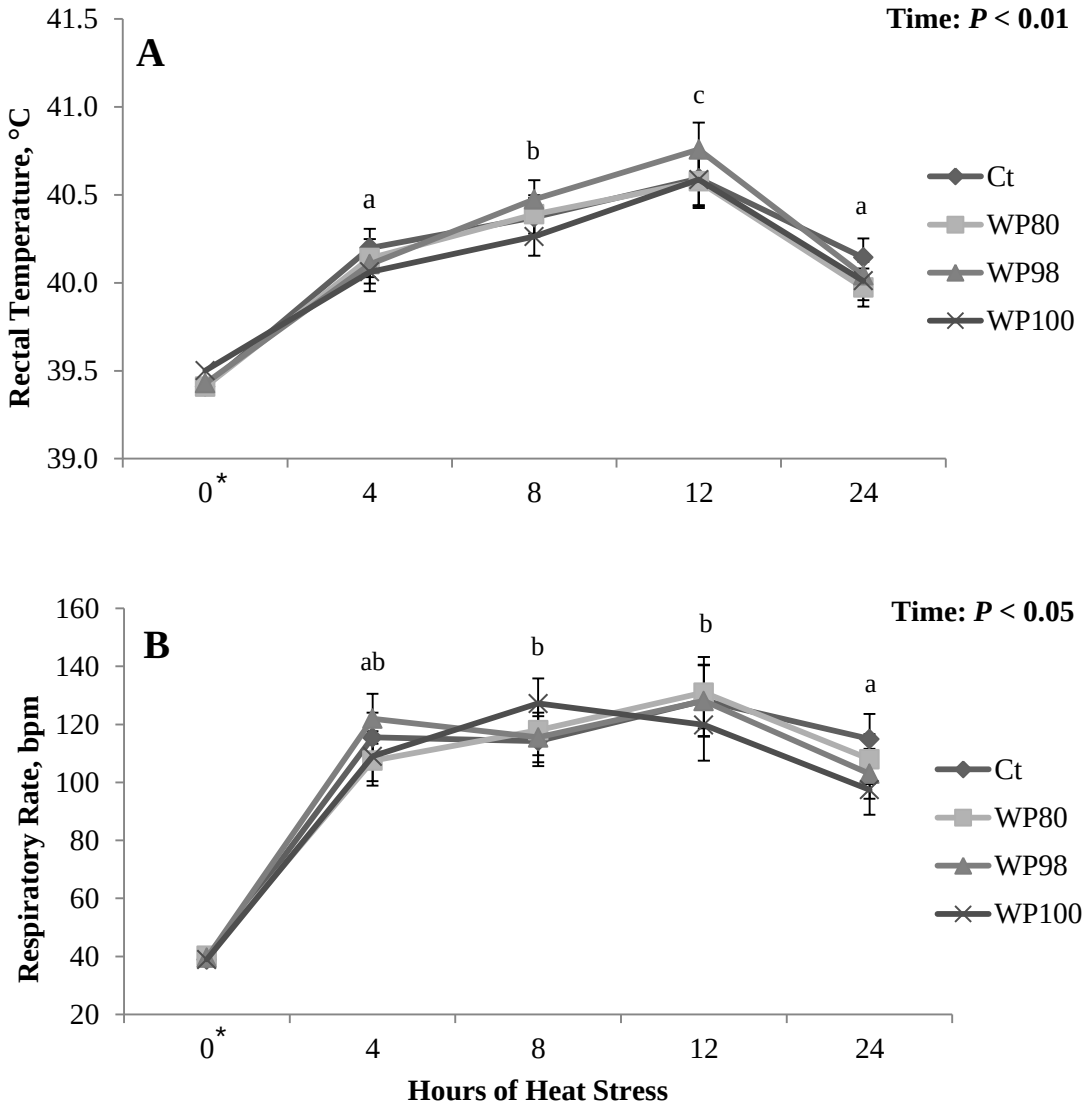

Figure 10. Effects of feeding diets containing no test product (Ct), or 80% milk whey protein (WP) + 20 % colostral whey protein (CWP; WP80), 98% WP + 2% CWP (WP98), 100% WP (WP100) test products on (A) rectal temperature and (B) respiratory rate of pigs exposed to constant heat stress conditions (32ºC) for 24 hours. [a,b,c] Represent differences between hours of heat stress ($P \leq 0.05$). *Represents the average values during period 1.

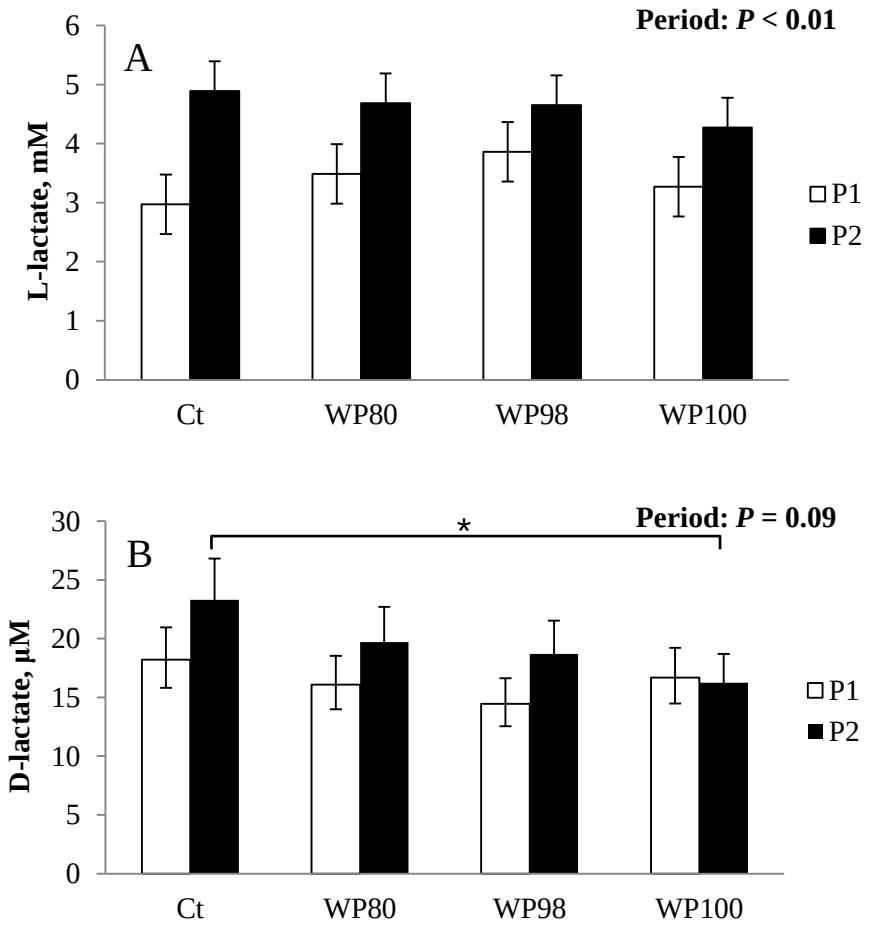

Figure 11. Effects of feeding diets containing no test product (Ct), or 80% milk whey protein (WP) + 20 % colostral whey protein (CWP; WP80), 98% WP + 2% CWP (WP98), 100% WP (WP100) test products on plasma (A) L-lactate, (B) D-lactate, and (C) lipopolysaccharide binding protein (LBP) concentrations of pigs during period 1 (P1; thermoneutral conditions: 19°C; ~46% humidity) and at the end of period 2 (heat stress conditions for 24 h: 32°C; ~26% humidity). *Represents differences between treatments during period 2 with period 1 as a covariate.

Table 14. Test dairy products nutrient composition

	Test dairy products		
	WP80	WP98	WP100
Protein, %	78.44	79.70	79.85
Fat, %	5.76	6.27	6.33
Lactose, %	7.53	6.70	6.58
Ash, %	2.93	2.88	2.88
Moisture, %	4.32	4.36	4.36

WP80 = test product containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP); WP98 = test product containing 98% WP + 2% CWP; WP100 = test product containing 100% WP

Table 15. Ingredients and formulated dietary nutrients

	Diet			
Parameter	Ct	WP80	WP98	WP100
Ingredients (%)				
Corn	73.51	84.27	84.27	84.27
Soybean meal (46.5)	22.30	5.75	5.75	5.75
Soybean oil	1.04	-	-	-
L-lysine HCl	0.3	0.2	0.2	0.2
DL-methionine	0.06	0.02	0.02	0.02
L-threonine	0.09	-	-	-
Monocalcium phosphate	1.14	1.16	1.16	1.16
Limestone	0.94	0.98	0.98	0.98
Salt	0.35	0.35	0.35	0.35
Vitamin Premix[1]	0.15	0.15	0.15	0.15
Trace Mineral Premix[2]	0.12	0.12	0.12	0.12
80% WP[3], 20% CWP[4]	-	7	-	-
98% WP, 2% CWP	-	-	7	-
100% WP	-	-	-	7
Nutrients				
ME – kcal/kg	1530	1533	1533	1533
Crude Protein %	16.9	15.3	15.3	15.3
SID[5] Lys %	0.97	0.97	0.97	0.97
SID Met + Cys %	0.56	0.56	0.56	0.56
SID Thr %	0.61	0.61	0.61	0.61
SID Trp %	0.16	0.20	0.20	0.20
Calcium %	0.65	0.66	0.66	0.66
Phos. % - total	0.60	0.55	0.55	0.55
Phos. % - avilable	0.30	0.31	0.31	0.31
Sodium %	0.16	0.16	0.16	0.16
Chlorine %	0.25	0.25	0.25	0.25

[1]Provided the following per kg of diet: vitamin A, 7,656 IU; vitamin D, 875 IU; vitamin E, 62.5 IU; vitamin K, 3.75 mg; riboflavin, 13.75 mg; niacin, 70 mg; pantothenic acid, 33.75 mg; vitamin B12, 62.5 µg.

[2]Provided the following per kg of diet: Fe, 121 mg as ferrous sulfate; Zn, 121 mg as zinc sulfate; Mn, 28.6 mg as manganese sulfate; Cu, 12.1 mg as copper sulfate; I, 0.22 mg as calcium iodate; Se, 0.22 mg as sodium selenite.

[3]Milk whey protein

[4]Colostral whey protein

[5]Standard ileal digestible

Table 16. Effects of dietary dairy products and environmental conditions on production parameters in pigs

	Trt					*P*	Contrast vs Ct		
Parameter	Ct	WP80	WP98	WP100	SEM	Trt	WP80	WP98	WP100
Period 1[1]									
ADG[2], kg/d	0.76	0.73	0.71	0.74	0.04	0.73	0.48	0.29	0.69
FI[3], kg/d	2.15	2.06	2.11	2.10	0.07	0.80	0.33	0.71	0.61
G:F[4]	0.36	0.35	0.34	0.35	0.02	0.73	0.61	0.27	0.51
Period 2[5]									
ΔBW[6], kg	-0.64	-0.43	-0.52	-0.41	0.33	0.96	0.66	0.80	0.63
ΔFI[7], kg	-0.90	-0.98	-0.93	-0.98	0.11	0.95	0.63	0.83	0.62

Ct = diet containing no test product; WP80 = diet containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP) test product; WP98 = diet containing 98% WP + 2% CWP test product; WP100 = diet containing 100% WP test product

[1]Thermoneutral conditions: 19°C; ~46% humidity

[2]Average daily gain

[3]Feed intake

[4]Gain to feed ratio

[5]Heat stress conditions for 24 h: 32°C; ~26% humidity

[6]Change in body weight (period 2 – period 1)

[7]Change in feed intake (period 2 – period 1)

Table 17. Effects of dietary dairy products on intestinal permeability parameters in 24 hour heat-stressed pigs

	Trt					*P*	Contrast vs Ct		
Parameter	Ct	WP80	WP98	WP100	SEM	Trt	WP80	WP98	WP100
Ileum									
TER[1]	142[bc]	90[a]	157[c]	104[ab]	16	0.02	0.03	0.51	0.10
FITC-Dextran APP[2]	17.2	20.5	15.2	23.2	8.5	0.91	0.77	0.84	0.63
Colon									
TER	90	89	89	100	7	0.66	0.87	0.94	0.34
FITC-Dextran APP	10.1	9.8	11.0	6.0	4.2	0.76	0.97	0.90	0.40

Ct = diet containing no test product; WP80 = diet containing 80% milk whey protein (WP) + 20% colostral whey protein (CWP) test product; WP98 = diet containing 98% WP + 2% CWP test product; WP100 = diet containing 100% WP test product

[1] Transepithelial electrical resistance, $\Omega \cdot cm^2$

[2] Fluorescein isothiocyanate labeled dextran apparent permeability coefficient, $\mu g/ml/min/cm^2$

CHAPTER 6: INTEGRATIVE SUMMARY

Heat stress (HS) jeopardizes both human health and animal agriculture productivity. Despite advances in barn design and cooling systems, HS is still a major economic burden during the warm summer months. Heat-induced revenue losses are mainly due to decreased production (decreased milk yield, egg laying, growth), altered carcass composition, depressed reproduction, increased veterinary cost, and mortality (St-Pierre *et al.* 2003; Baumgard and Rhoads 2013). Moreover, productivity improvements achieved through genetic selection likely compromises thermotolerance, as increased lean accretion, milk yield, etc. are associated with an increase in metabolic heat production (Brown-Brandl *et al.* 2003).

Heat waves represent the environmental hazard with the highest human mortality (Changnon *et al.* 1996). Treatment protocols against heat-related illnesses are restricted to cooling and re-hydration, which illustrates the current knowledge gap in the pathophysiology of these diseases (Leon and Helwig 2010a). Further, the inability to anticipate the long term consequences after heat exposure is likely the biggest limitation to proper treatment, as deaths frequently occur months and even years after (Bouchama and Knochel 2002). For both human health and animal agriculture, heat-related issues will be likely exacerbated as a sustained rise in earth's temperature and an increase in the frequency of heat waves have been predicted (Luber and McGeehin 2008). Therefore, it is imperative to understand the biology of the adaptation to HS in order to develop more effective treatment protocols and mitigation strategies.

In the current dissertation we have demonstrated that HS induces profound changes in energetic metabolism that are independent of the reduction in feed intake (FI) exhibited by hyperthermic pigs. During periods of negative energy balance (e.g. lowered plane of nutrition, increased energetic demand), changes in post-absorptive metabolism occur to support a dominant

physiological state (e.g. skeletal muscle and milk synthesis) and ultimately sustain those tissues that are obligate glucose utilizers (e.g. brain and red blood cells; Bauman and Currie 1980). Specifically, adipose tissue is mobilized to provide non-esterified fatty acids (NEFA) as an alternative fuel and peripheral insulin sensitivity decreases in order to spare glucose. This is exactly what we observed in our pair-fed thermoneutral (PFTN) controls which, upon feed restriction initiation, acutely increased plasma NEFA concentration (chapters 2 and 3) and decreased whole-body insulin sensitivity (chapter 3).

In contrast and despite a similar reduction in FI, HS pigs did not mobilize as much adipose tissue, as demonstrated by the decrease in circulating NEFA concentration (chapter 2 and 3) compared to PFTN controls. Moreover, HS pigs had a blunted NEFA response to an epinephrine challenge even when compared to TN *ad libitum* conditions (chapter 2). This likely explains the results of previous studies where animals reared under HS have increased adiposity (Baumgard and Rhoads 2013). The effects of HS on lipolysis might be partially controlled at the transcriptional level, as adipose tissue triglyceride lipase (a key enzyme of the lipolytic cascade; *ATGL;* Zimmermann *et al.* 2004) gene expression was decreased (chapter 2). Further, the gene encoding the AMPK (the so-called cellular energy gauge) regulatory subunit (*PRKAG1*) was also down-regulated, which is interesting as AMPK has been implicated in the control of ATGL content and activity (chapter 2; Gaidhu *et al.* 2012).

We hypothesized that altered insulin homeostasis might be responsible for the lack of adipose tissue mobilization observed during HS. Insulin is a potent antilipolytic signal and is frequently elevated in HS animals when compared with PFTN counterparts (Baumgard and Rhoads 2013). However, several findings in the current dissertation appear to dispute this hypothesis. First, basal plasma NEFA was consistently decreased for the length of the

environmental exposure in HS animals, while circulating insulin/C-peptide was only increased during early stages of HS (chapter 2 and 3). Second, the decrease in circulating NEFA in response to a hyperinsulinemic clamp was numerically blunted during HS and compared to period 1 which might suggest that insulin was not as effective inhibiting lipolysis (chapter 3). Third, we did not detect differences in the abundance of adipose tissue insulin signaling markers in response to an insulin tolerance test (chapter 3). Fourth, adipose tissue insulin receptor (*INSR*) transcript abundance was decreased during HS (chapter 2). Overall, these findings might indicate that the lack of NEFA mobilization is not due to increased insulin signaling at the adipose tissue level. As discussed in chapter 3, even if insulin signaling is not increased, insulin action might be still enhanced by other metabolites like lactate. For instance plasma lactate, which is increased in a variety of HS models including ours (chapter 5), mediates insulin antilipolytic effects by interacting with the G protein-coupled receptor 81 (Ahmed *et al.* 2010). In addition, other heat-induced signals like prolactin might partially mediate the blunted lipolytic response observed during HS (LaPensee *et al.* 2006; Brandebourg *et al.* 2007). Moreover, we observe a sharp decrease in circulating thyroid hormones during HS (chapter 2). Thyroid hormones are well correlated with whole-body energy expenditure and heat production and are known to stimulate lipolysis and NEFA utilization (Mullur *et al.* 2014). Therefore, the heat-induced reduction in adipose tissue mobilization might be the result of decreased thyroid hormones' stimulation. Interestingly, hypothyroid humans have reduced sensitivity to adrenergic signals (Mullur *et al.* 2014), which may explain why HS pigs had a blunted NEFA response to epinephrine (chapter 2). Nonetheless, the lack of adipose tissue mobilization is one of the most consistent findings during HS across species and models, which suggests that it is a key component of HS adaptation.

Despite its unclear role in lipid metabolism during HS, it is still evident that proper insulin action is crucial for successfully adapting to a heat load, as demonstrated by the increased mortality among diabetics during a heat wave (Schuman 1972; Semenza *et al.* 1999). Increased basal and glucose stimulated circulating insulin were consistent findings in previous studies in HS ruminants (Baumgard and Rhoads 2013). However, we have obtained conflicting results in pigs (chapter 2 and 3). The variability between species might be due to differences in insulin sensitivity. Ruminants are intrinsically more insulin resistant due to their reliance on gluconeogenesis for glucose synthesis as dietary glucose is fermented by ruminal microorganisms (Brockman and Laarveld 1986). Thus, ruminants might require an increase in circulating insulin to attain an increase in insulin action; while in pigs, which are more insulin sensitive, changes in peripheral insulin signaling might be adequate for insulin to exert its effects on HS adaptation. As mentioned before, the metabolic adaptation to feed restriction includes the instauration of peripheral insulin resistance as a glucose sparing mechanism (Bauman and Currie 1980). Herein, we demonstrate that, despite a marked reduction in FI, HS increases whole-body insulin-stimulated glucose uptake relative to PFTN (chapter 3). The increase in insulin action explains the frequently observed hypoglycemia (chapter 2), as well as the increase in glucose utilization described in the literature during HS (Fink *et al.* 1975; Febbraio 2001). Nonetheless, the relative contribution of the different tissues to glucose uptake remains unknown. Based on sheer mass, skeletal muscle is likely a major glucose sink. In agreement, we report an increase in the abundance of muscle insulin signaling markers during HS (chapter 3). However, the immune system might be the fate of a substantial amount of glucose. We and others have demonstrated that HS decreases intestinal barrier function, increases intestinal permeability, and subsequently elevates circulating LPS (Hall *et al.* 2001; Lambert 2004; Pearce *et al.* 2013c). Likewise, in the

current dissertation we discovered an increase in intestinal permeability to bacterial components and immune system activation during HS. Heat-stressed pigs had increased plasma D-lactate concentrations, an indicator of microbial metabolism (chapter 5). In addition, HS increased CD14 gene expression in skeletal muscle and circulating lipopolysaccharide binding protein (LBP; chapter 2), supporting the activation of the toll-like receptor 4 (TLR4) pathway and stimulation of an acute phase response. Interestingly, once activated, immune cells become obligate glucose utilizers capable of oxidizing a substantial amount of glucose (Greiner *et al.* 1994; Maciver *et al.* 2008). Thus, further research is required in order to elucidate the relative contribution of different tissues to whole-body glucose utilization during HS.

Heat stress not only affects substrate utilization, also seems to alter cellular energetics as there is evidence for hyperthermia to increase aerobic glycolysis at the expense of oxidative phosphorylation (Baumgard and Rhoads 2013). In agreement, HS increases circulating lactate (chapter 5) and down-regulates the transcript abundance of tricarboxylic acid (TCA) cycle and electron transport chain enzymes (chapter 2). This resembles the Warburg effect utilized by rapid proliferating cells, like cancer and activated immune cells, but the reasons why this occurs during HS remain unknown cells (Warburg 1956; Kim and Dang 2006). Maximum ATP is generated by full oxidation of glucose, but aerobic glycolysis occurs at a much faster rate and can substantially contribute to cell energetics. Further, aerobic glycolysis might supply building blocks like lactate or alanine (Berg *et al.* 2002) that can be utilized for glucose production in other tissues like the liver via the Cori cycle, as HS has been reported to increase hepatic glucose output by others (Febbraio 2001).

The physiological reasons behind an increase in insulin action during HS might reside on insulin's role as a heat shock protein (HSP) inductor, which could explain why diabetics have

decreased skeletal muscle HSP abundance (Li *et al.* 2006). Reciprocally, the induction of HSP might be responsible for the increase in insulin sensitivity observed during HS, as demonstrated by the thermal therapy literature (Hooper 1999; Gupte *et al.* 2009). Further, the lack of adipose tissue mobilization might also contribute to the heat-induced increase in insulin sensitivity relative to PFTN controls, as increased circulating NEFA have been linked to insulin resistance (Johnson and Olefsky 2013). Ultimately, the shift in substrate utilization might represent an attempt to reduced metabolic heat production as oxidizing glucose in order to generate ATP is more efficient in a per mole of oxygen basis than oxidizing fatty acids (Gerald and Friedman 1998).

The current dissertation demonstrates that HS has profound effects in metabolism that would partially explain the phenotypic changes observed in animals reared under hyperthermic conditions. Our results open several lines for future research. Firstly, due to its implications on whole-body energy expenditure, thyroid hormones' role on the HS phenotype deserves further investigation. Our data on hepatic 5'-deiodinase activity suggests a decrease in peripheral T_4 to T_3 transformation. However, fasting stimulates the transition from T_3 to reverse T_3 (an inactive form; Palmblad *et al.* 1977). Whether this mechanism is exacerbated during HS is of interest. Further, circulating thyroid hormones are centrally regulated by negative feedback mechanisms. The effects of HS on thyroid hormone's central regulation and its consequences on the heat-induced phenotype (e.g. reproduction) remain unknown. Moreover, the role of systemic inflammation in the pathophysiology of HS and heat-related illnesses also remains unclear. Our results provide evidence of increased intestinal permeability and immune system activation: increased circulating D-lactate and LBP (chapters 5 and 2), increased skeletal muscle CD14 transcript abundance (chapter 2), and increased ovarian TLR4 protein abundance (Nteeba et al.,

unpublished). However, recent data failed to detect signs of inflammation based on classical markers like cytokines (Pearce *et al.* 2013b; Rosado Montilla *et al.* 2014). This is not surprising as the heat stroke literature describes the so-called cytokine storm lagging behind the heat exposure (Leon 2007). Regardless, further investigation would be necessary in order to elucidate the immunes system's contribution to whole-body energetics during HS. From a practical point of view, understanding the biology of the adaptation to a heat load allows for developing of mitigation strategies and treatment protocols against specific aspects regarding HS consequences on health and productivity. Herein, we describe the feasibility of nutritional management to mitigate the deleterious effects of HS on intestinal permeability. Advantages of nutritional interventions over long-term strategies, like genetic selection, include immediate and flexible implementation (application can be limited to the warm summer months or when expecting a heat wave), potential applicability across species and therefore valuable to a variety of industries, and amenable to diverse production systems. Nevertheless, further research at a bigger scale and under standard production settings is required in order to determine whether ameliorating intestinal barrier function ultimately translates into production improvements during environmental hyperthermia.

In conclusion, HS jeopardizes human health and livestock welfare and productivity. Changes in metabolism and whole-body energetics are crucial for successfully adapting to a heat load. A thorough understanding of the triggers as well as the biological meaning of such changes is critical in order to develop targeted treatments against HS and heat-related consequences. The use of the pig as a model has the potential to impact both human medicine and animal agriculture fields. In this dissertation we demonstrate that HS blunts both basal and epinephrine-stimulated adipose tissue mobilization. Further, we proved that HS pigs exhibit an

increase in whole-body insulin-induced glucose-uptake compared to TN controls in a similar plane of nutrition. Moreover, we confirmed the viability of supplemental dietary zinc amino complex on to mitigate the negative effects of heat stress on intestinal health. Finally, we open several lines of investigation for further research.

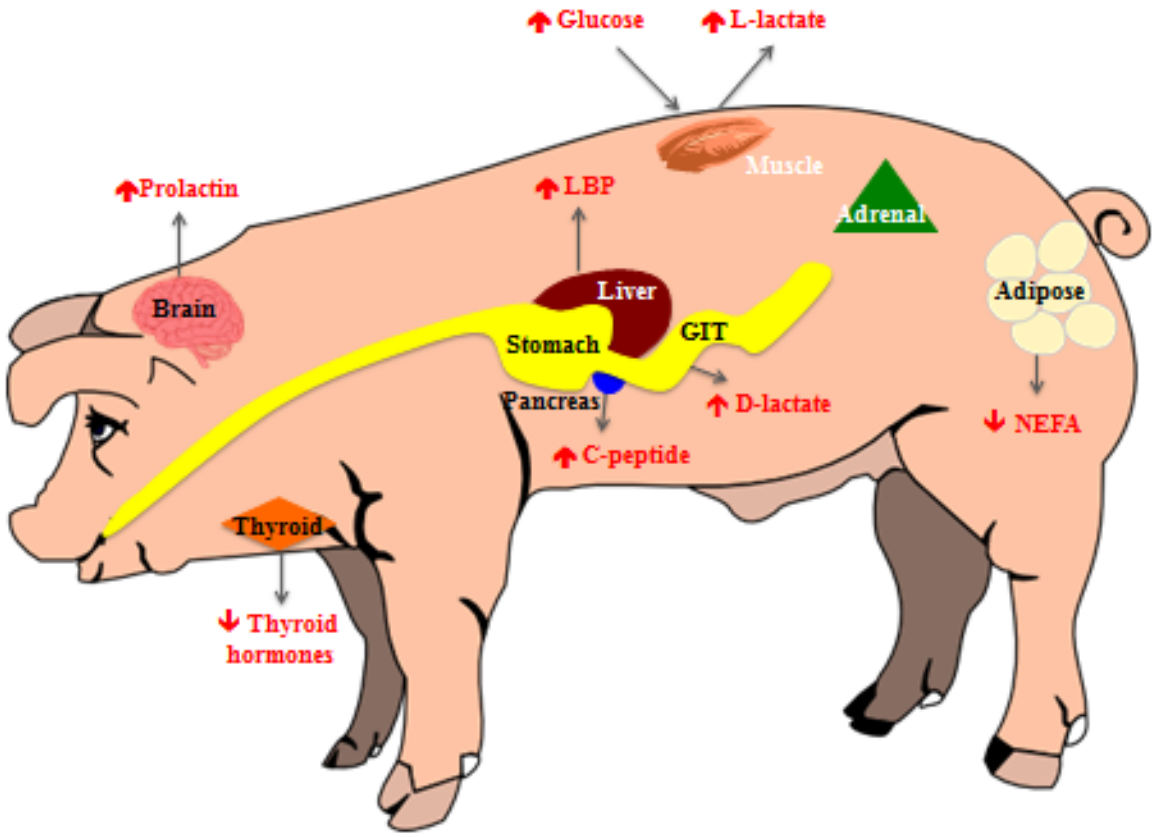

Figure 12. Effects of heat stress on plasma metabolites in growing pigs discovered or confirmed in the current dissertation.

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APPENDIX A: EFFECTS OF HS ON VIABILITY, PROLIFERATION AND INSULIN SECRETION IN β-TC-6 CELLS

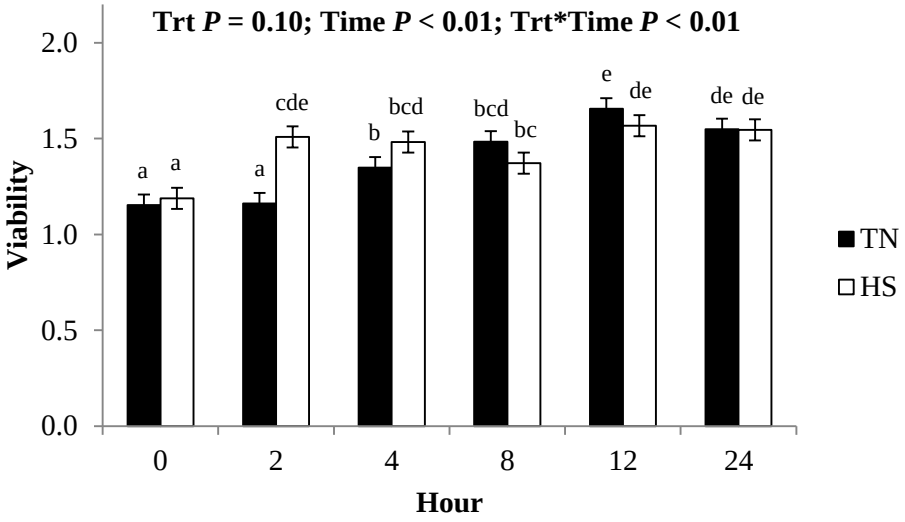

Figure 13. Temporal effects of heat stress on cellular viability in β-TC-6 cells. Cells from passages 15-18 were exposed to thermoneutral (TN; 37°C, 5% CO_2) or heat stress (HS; 41°C, 5% CO_2) conditions. Viability was determined as the cell-mediated reduction of resazurin to resorufin (CellTiter-Blue®, Promega, Madison, WI). Experiments were replicated 3 times. [a-e]Means with different superscripts differ ($P \leq 0.05$).

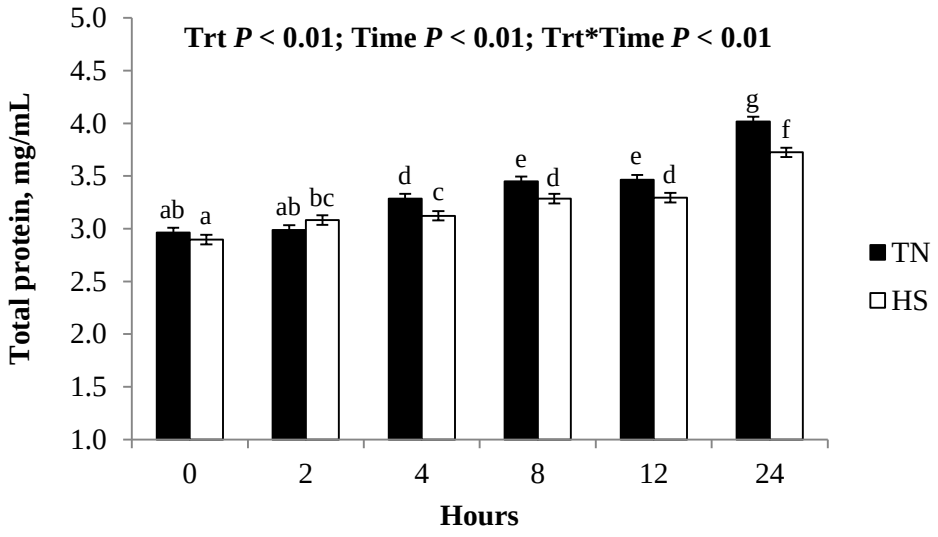

Figure 14. Temporal effects of heat stress on total protein content in β-TC-6 cells. Cells from passages 17-20 were exposed to thermoneutral (TN; 37°C, 5% CO_2) or heat stress (HS; 41°C, 5% CO_2) conditions. After two washes with phosphate buffer saline, cells were collected and total protein extracted in lysis buffer. Total protein content was determined with the bicinchonic acid assay (BCA, Pierce™, Thermo Fisher Scientific Inc., Rockford, IL). Experiments were replicated 3 times. $^{a\text{-}f}$Means with different superscripts differ ($P \leq 0.05$).

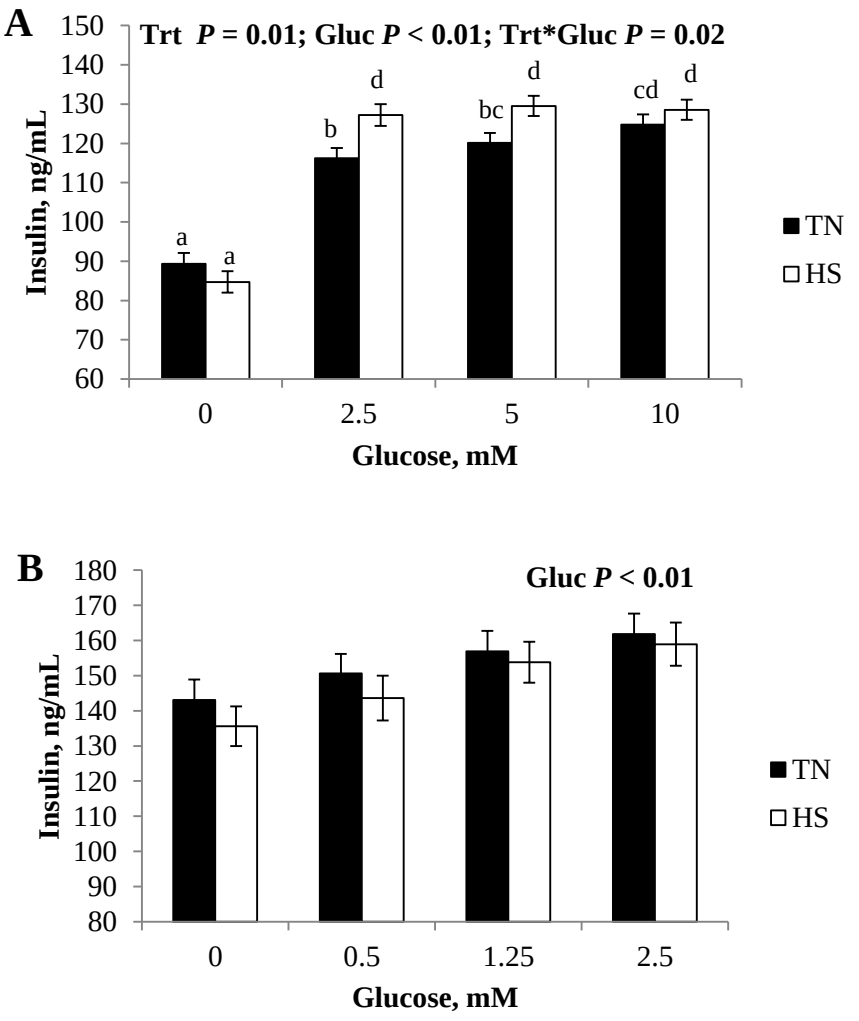

Figure 15. Effects of heat stress on glucose stimulated insulin secretion in β-TC-6 cells. Cells from passages 3-15 were exposed to thermoneutral (TN; 37°C, 5% CO_2) or heat stress (HS; 41°C, 5% CO_2) conditions for 4 hours. During the environmental exposure, cells were washed twice (30 minutes per wash) with 0 mM glucose Krebs-Ringer bicarbonate buffer followed by a 2 hours incubation in incremental concentrations of glucose: (A) 0-10 mM glucose, and (B) 0-2.5 mM glucose. After 2 hours of glucose stimulation, media was collected for insulin determination. The number of alive cells were counted using Trypan Blue staining and used as a covariate. Experiments were replicated 3 times. $^{a\text{-}e}$Means with different superscripts differ ($P \leq 0.05$).

APPENDIX B: MISCELLANEOUS

Table 18. Effects of pair-feeding or heat stress on plasma amino acids' concentrations in growing pigs

	Period 1[1]		Period 2[2]			*P*		
Amino acids, μM	PFTN	HS	PFTN	HS	SEM	Trt[3]	Period	Trt*Period
3-Methylhistidine	18.8	16.7	17.6	16.7	2.0	0.56	0.64	0.64
Alanine	311.8	337.7	298.0	327.8	14.0	0.14	0.19	0.83
β-Alanine	7.4^{b}	6.6^{ab}	4.6^{a}	9.3^{b}	0.9	0.06	0.94	0.01
α-Aminobutirate	24.8	25.4	30.0	30.3	2.2	0.89	<0.01	0.92
γ-Aminobutirate	50.9	42.3	45.5	40.4	4.0	0.17	0.25	0.57
Arginine	112.2^{ab}	130.3^{bc}	139.4^{c}	110.8^{a}	7.3	0.59	0.33	<0.01
Asparagine	47.3	62.2	50.2	63.5	3.2	<0.01	0.52	0.81
Aspartate	10.5	16.1	9.0	15.6	2.5	0.05	0.66	0.82
Carnitine	17.2	16.3	16.6	18.5	1.7	0.77	0.62	0.40
Citrulline	59.4^{a}	67.2^{ab}	78.0^{b}	56.0^{a}	5.6	0.31	0.39	<0.01
Cysteine	53.7	59.1	58.2	72.2	4.6	0.09	0.04	0.28
Glutamate	139.2^{b}	186.1^{c}	122.5^{a}	154.8^{b}	14.7	0.07	<0.01	0.07
Glutamine	516.0	616.8	553.5	608.2	18.5	<0.01	0.43	0.21
Glycine	644.2	762.8	783.7	894.6	44.0	0.04	<0.01	0.91
Histidine	77.8^{a}	90.7^{b}	77.6^{a}	103.0^{c}	4.2	<0.01	0.12	0.11
Isoleucine	87.1^{a}	108.4^{b}	79.0^{a}	121.8^{b}	6.1	<0.01	0.64	0.07
Leucine	167.5	218.5	173.0	223.9	11.0	<0.01	0.57	0.99
Lysine	55.6^{a}	57.3^{a}	64.6^{a}	111.3^{b}	11.1	0.09	<0.01	0.02
Methionine	30.0^{a}	36.8^{b}	35.9^{b}	51.2^{c}	2.9	0.01	<0.01	0.05
Ornithine	25.6^{a}	41.3^{b}	39.7^{b}	31.5^{ab}	4.3	0.38	0.63	0.02
Phenylalanine	74.1^{a}	79.6^{a}	80.6^{a}	100.9^{b}	4.4	0.03	<0.01	0.05
Proline	211.8^{a}	276.2^{b}	293.4^{b}	294.2^{b}	20.7	0.17	0.02	0.11
Serine	87.9	112.9	85.9	125.7	7.4	<0.01	0.44	0.30
Taurine	58.7^{ab}	49.4^{a}	58.9^{ab}	62.2^{b}	5.1	0.65	0.06	0.07
Threonine	127.5	171.9	143.2	174.1	9.3	0.01	0.18	0.31
Tryptophan	61.0	67.9	73.0	87.9	3.7	0.01	<0.01	0.26
Tyrosine	84.8^{a}	114.7^{b}	112.0^{b}	109.8^{b}	5.8	0.07	0.02	<0.01
Valine	180.7^{a}	232.1^{b}	174.1^{a}	283.6^{c}	11.8	<0.01	0.07	0.02

[1]All pigs were fed *ad libitum* in thermoneutral conditions (20°C).
[2]Pigs were either fed *ad libitum* in heat stress conditions (HS, 32°C) or pair-fed in thermoneutral conditions (PFTN) for 5 days
[3]Treatment

Table 19. Effects of catheterization and heat stress on complete blood count in growing pigs

	Pre-catheterization[1]		Post-catheterization[2]		Environmental exposure[3]			*P*		
Parameter	PFTN	HS	PFTN	HS	PFTN	HS	SEM	Trt	Day	Trt*Day
White blood cells, x10^3/µl	11.71^{x}	12.36^{x}	14.11^{y}	15.74^{y}	15.74^{y}	15.25^{y}	0.87	0.59	<0.01	0.08
Neutrophils, x10^3/µl	$4.60^{y,c}$	$3.68^{y,bc}$	$1.59^{x,a}$	$3.64^{x,bc}$	$4.10^{y,bc}$	$3.35^{y,b}$	0.39	0.76	<0.01	<0.01
Lymphocytes, x10^3/µl	6.37^{x}	7.70^{x}	10.75^{y}	10.11^{y}	10.14^{y}	10.24^{y}	0.54	0.64	<0.01	0.08
Monocytes, x10^3/µl	$0.503^{x,a}$	$0.697^{x,ab}$	$0.712^{y,ab}$	$1.223^{y,c}$	$0.806^{y,b}$	$0.860^{y,b}$	0.098	0.03	<0.01	0.01
Eosinophils, x10^3/µl	0.067^{x}	0.057^{x}	0.852^{y}	0.530^{y}	0.668^{y}	0.607^{y}	0.121	0.31	<0.01	0.20
Basophils, x10^3/µl	0.050^{a}	0.088^{b}	0.058^{ab}	0.085^{ab}	0.064^{ab}	0.052^{a}	0.013	0.26	0.24	0.04
Unidentified, x10^3/µl	0.120	0.138	0.145	0.143	0.174	0.150	0.042	0.94	0.74	0.88
Red blood cells, x10^6/µl	6.45	6.29	6.41	6.22	6.82	6.18	0.20	0.15	0.47	0.31
Hemoglobin, g/dL	10.78	10.95	10.70	10.85	11.31	10.80	0.27	0.79	0.57	0.38
Hematocrit, %	34.63	35.27	34.08	34.53	35.96	34.15	0.89	0.76	0.63	0.33
Mean corpuscular volume, fl	53.68^{z}	56.25^{z}	53.22^{y}	55.63^{y}	52.87^{x}	55.35^{x}	0.58	0.01	<0.01	0.82
Mean corpuscular hemoglobin (MCH), pg	16.72	17.48	16.72	17.47	16.67	17.53	0.22	0.02	0.99	0.75
MCH concentration, g/dL	31.13	31.10	31.43	31.38	31.52	31.65	0.23	0.96	<0.01	0.68
Red cell distribution width[7], %	15.67	16.12	15.72	16.05	15.38	15.88	0.26	0.26	<0.01	0.22
Platelets, x10^3/µl	423^{b}	418^{b}	336^{a}	439^{b}	411^{b}	410^{ab}	27.00	0.29	0.24	0.02
Mean platelet volume[8], fl	$8.77^{x,a}$	$8.50^{x,a}$	$10.02^{y,b}$	$8.60^{y,a}$	$8.56^{x,a}$	$8.43^{x,a}$	0.31	0.15	<0.01	<0.01

[1]Blood samples were obtained during the catheterization surgery. All pigs were in thermoneutral conditions (20°C) and fed *ad libitum*.

[2]Blood samples were obtained 2 days after the catheterization surgery. All pigs were in thermoneutral conditions and fed *ad libitum*.

[3]Blood samples were obtained after 3 days of *ad libitum* feed intake in constant heat stress conditions (HS; 32°C) or pair-feeding in thermoneutral conditions (20°C, PFTN).

[x-z]Days with different letters differ ($P \leq 0.05$)

[a,b]Means with different letters differ ($P \leq 0.05$)

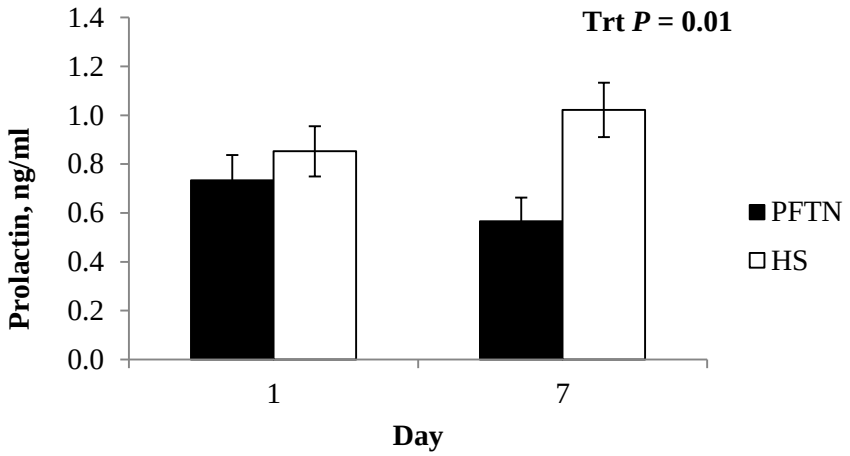

Figure 16. Effects of *ad libitum* feed intake in constant heat stress conditions (HS; 36°C) and pair-feeding in thermoneutral conditions (PFTN; 19°C) on temporal changes in plasma prolactin concentration in growing pigs.

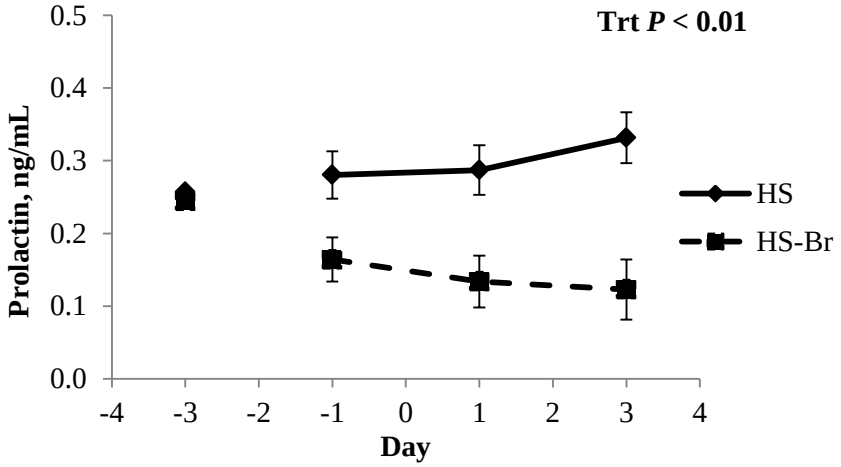

Figure 17. Effects of heat stress and bromocriptine treatment on plasma prolactin concentration in growing pigs. Pigs were exposed to thermoneutral conditions (20°C) until the initiation of heat stress conditions (constant 32°C) on day 0. Pigs were treated twice daily from day -2 with either bromocriptine (HS-Br; IV 0.5 mg/kg/day) or vehicle (HS). Plasma prolactin concentrations prior to bromocriptine and environmental treatment (day -3) were used as a covariate.